THE USE OF IN VITRO KERNEL DEVELOPMENT FOR THE EXAMINATION OF STARCH BIOSYNTHESIS IN MAIZE

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Starch blosynthesis in the maize endosperm was exsmined by the use of *in vitro* kernsl developmest. It was shown thst kernels of wild type as well as the starch deflclent mutsnts shrunken-1 (sh1) and shrunken-2 (sh2), would develop to meturlty when plsced in culture on medlum contslnlng sucrose st 5 dsys post-pollinstion. At meturity the kernels had their expectsd phenotypes. Analysis of the enzymes sucrose synthetsse, sdenosine diphospho-glucose pyrophosphorylase snd starch synthetsse throughout development revesled thelr activities to be simllsr to fleld grows maize. Sugsrs and starch levels throughout development, ss well as germinstion of kernels grown *in vitro*, were slmilsr to kernels grown *in vivo*.

After establishing that growth *in vitro* was comparable to growth *in vivo*, experiments were performed to determine if the *in vitro* kernel development system would be useful in investigating starch biosynthesis. Kernels of wild type, *sh1* and *sh2* were grown on sucrose, glucose or fructose. Regardless of the carbon source, sucrose was present in the endosperms of all three genotypes. Also, *sh1* and *sh2* kernels accumulated sucrose when grown on reducing sugars indicating that sucrose is synthesized in endosperm cells. The buildup of sucrose in *sh1* may indicate that sucrose synthetase does not synthesize sucrose.

Wild type and *sh2* kernels had significantly greater weights and starch content when grown on sucrose whereas *sh1* kernels had a significantly greater weight and starch content when grown on glucose. There was a correlation between seed vigor and starch content.

The uptake and conversion of [^{14}C] sucrose and glucose was investigated. Results indicate that 55% of the sucrose was transported into the maize endosperm of the three genotypes without hydrolysis. The uptake of labeled sucrose was lowest in *sh1* whereas the uptake of labeled glucose was similar in all three genotypes. Also, the conversion of sucrose into other compounds was least in *sh1*. These results are consistent with the notion that the

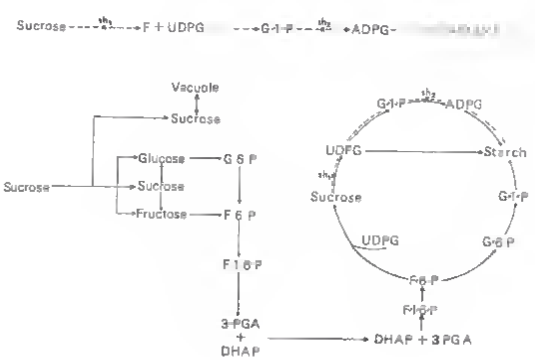
function of sucrose synthetase is in the breakdown of sucrose.

INTRODUCTION

The starch biosynthetic pathway in maize has been investigated in detail by the use of physiological and genetic analysis. Biochemical investigations have identified plant enzymes capable of converting sucrose, through several intermediates, into starch. From these studies, pathways have been proposed for the flow of carbon from sugar to starch (Figure 1). The reactions shown in Figure 1 are known to occur in the maize endosperm cells and have all been implicated as integrally involved in starch biosynthesis. Because it has not been possible, by physiological means, to determine which sequences of reactions are important to starch biosynthesis, the specific pathway remains unclear.

It has been possible, by genetic analysis, to determine the involvement of a limited number of enzymes in starch biosynthesis. Mutants have been isolated that are characterized by alterations in the amount or quality of

Figure 1. Reaction sequences leading to starch biosynthesis in the maize endosperm. The reactions in the upper portion are based on genetic evidence. The sequence of reactions in the bottom portion are based on physiological data. The reactions outlined in the lower portion is a modification of a scheme by Shannon (1978). UDPG= UDPG pyrophosphorylase, ADPG= ADPG pyrophosphorylase, DHAP= dihydroxyacetone phosphate, 3-PGA= 3-phosphoglycaric acid.


Sucrose
F + UDPG
G-1-P
ADPG
Vacuole
Sucrose
Glucose
G 6 P
Sucrose
Sucrose
Fructose
F 6 P
F 1,6 P
3-PGA
+
DHAP
DHAP + 3PGA
G-1-P
ADPG
UDPG
Starch
Sucrose
G-1-P
UDPG
G-6-P
F-6-P
F-1,6-P

starch in the endosperm tissue. These have been investigated to determine if an enzyme deficiency can be correlated with the alteration in starch. A deficiency of an enzyme is strong but not definitive evidence that the enzyme has an important and integral role in starch biosynthesis.

By the use of these techniques, enzymes were identified that appear to be vital for normal starch biosynthesis to occur. These enzymes include sucrose synthetase, ADPG pyrophosphorylase and starch synthetase which will be discussed in detail below. From these data a pathway has been proposed and is shown in the top portion of Figure 1 and as the double lines in the bottom portion of the figure.

The flow of carbon from sucrose to starch includes more than the biochemical steps that are outlined in Figure 1. The movement of sucrose from the phloem to the endosperm cells and conversions of sucrose that may occur must also be considered. The structure of the basal area of the kernel has been examined by Kiesselbach and Walker (1952), Weatherwax (1930), Kiesselbach (1949) and more recently by Felker and Shannon (1980). The vascular bundles terminate below the kernel base where the carpel and ovule tissue fuse (Kiesselbach 1949). Above the vascular tissue is a region consisting of parenchyma cells

from which the closing layer will form (Kiesselbach and Welker 1952). Above this is the placento-chalazal region consisting of parenchyma cells which by 20 days post pollination (20dpp) are dead (Felker and Shannon 1980). Above the placento-chalazal region are the remnants of the nucellar tissue and adjacent to this area are the basal cells of the endosperm. The basal cells of the endosperm are characterized by cell wall ingrowths consistent with their proposed function as transfer cells (Felker and Shannon 1980, Wetherwax 1930).

The movement of sucrose into maize kernels has been extensively studied by Shannon and co-workers. They found that, after supplying [^{14}C] labeled CO_2 to maize leaves, radioactive sugars and starch could be recovered from the endosperm tissue (Shannon 1968,1974, Shannon, Creech and Loerch, 1970). One hour after exposure to labeled CO_2, reducing sugars accounted for greater than 80% of the radioactivity whereas sucrose accounted for the majority of the label after 5 hr. (Shannon 1968). This suggests that sucrose was hydrolyzed during uptake into the kernels and that it was resynthesized after entry.

In an analysis of the distribution of radioactivity in kernel segments, Shannon (1972) found that most of the labeled reducing sugars were located in the placento-chalazal region suggesting that sucrose hydrolysis

occurred in this region. At later time intervals, sucrose was found to be labeled but reducing sugars still accounted for most of the label in the placento-chalazal region. Shannon and Dougherty (1972) showed that invertase was present in the basal area of the endosperm tissue whereas sucrose synthetase was not found, implying that only invertase was responsible for the breakdown of sucrose in this region.

From these results Shannon (1978) has suggested that hydrolysis of sucrose in the kernel base is followed by resynthesis of sucrose in the endosperm tissue. Recently Liu and Shannon (1981a,b) have suggested, based on metabolite compartmentalization, that resynthesis of sucrose occurs in the amyloplast.

In wheat endosperms however, sucrose hydrolysis does not occur during uptake. In a series of *in vitro* experiments Jenner (1973,1974) exposed detached wheat ears or isolated grains to solutions containing sucrose in which the fructosyl moiety was labeled with [^{14}C]. Isolation of sucrose from the endosperm tissue showed that the majority of the label was still in the fructosyl moiety indicating uptake without hydrolysis. An advantage of this system in contrast to the work of Shannon is that Jenner was able to use an *in vitro* system and thus exercise more control over the uptake experiments

After movement of the sucrose into the endosperm cells, the next series of events is the enzymatic conversion of sucrose into starch. The first enzyme, for which genetic data are available and that is considered to be involved in the conversion of sucrose to starch, is sucrose synthetase which catalyzes the reaction:

SUCROSE + UDP <------> UDPG + FRUCTOSE

This enzyme is thought to be involved in the initial breakdown of sucrose for several reasons.

First, the finding that the starch mutant shrunken-1 (sh1) (Chourey & Nelson 1976) has low levels of sucrose synthetase is taken as evidence that this enzyme is important in starch synthesis. Secondly, Tsai, Salamini and Nelson (1970) and Hawker (1971) have shown that the activity of sucrose synthetase in the maize endosperm coincides with the time of rapid starch synthesis. In contrast, activity of invertase peaked before the time of rapid starch synthesis. In growing potato tubers (Pressy 1969), rice grains (Perez et al 1975) and barley grains (Baxter and Duffus 1973) the level of sucrose synthetase is much greater than the level of invertase during the period of active starch synthesis.

Sucrose synthetase has been isolated from several sources. Sucrose synthetase is a tetramer with a molecular weight estimated to be from 290,000 in potato (Pressey 1969), to 400,000 in rice (Nomura and Akazawa 1973) and bean (Delmar 1972a). It has been reported to behave as if it were membrane bound in bean (Delmar and Albersheim 1970) and has been localized in the cytoplasm of castor bean endosperm (Nishimura and Beevers 1979). Little is known about its regulation although it has been shown to be affected by auxin in bean (Delmar 1972b).

Of particular interest has been the question of the specific role of sucrose synthetase. The reaction catalyzed by sucrose synthetase is easily reversible so that the role of sucrose synthetase could be in the formation or degredation of sucrose. The synthetic and degradative functions of the enzyme have different pH optima in rice (Nomura and Akazawa 1973), sorghum (Sharma and Bhatia 1980), potato (Pressey 1969) and maize (Chourey & Nelson 1979) which may suggest a control of activity by pH.

Evidence suggests that the function of sucrose synthetase is in the degradation of sucrose in maize. Chourey (1971) found interallelic complementation in sh1 mutants that lead to a wild type phenotype. Analysis of these alleles revealed that one allele, in a homozygous

condition, had 30% of the wild type enzyme level in the synthetic direction but only 4.5% in the cleavage direction (Chourey and Nelson 1975). When wild type levels of starch were obtained by intraallelic complementation, enzymatic activity in the synthetic direction decreased slightly whereas the activity in the direction of sucrose cleavage doubled in comparison to that found in the alleles when homozygous. These data indicate that the role of sucrose synthetase is in the degradation and not synthesis of sucrose.

Other evidence also points to the function of sucrose synthetase in the breakdown of sucrose. Pressey (1969) has reported that the cytoplasmic pH favors sucrose degradation in potato The concentration of hexose monophosphate falls during the period of rapid starch synthesis in pea which has been suggested to favor the breakdown of sucrose (Rowan and Turner 1957). In the pea epicotyl, the area which is characterised by high levels of sucrose synthetase also has higher levels of fructose which may be expected if breakdown is favored (Maclachlon et al. 1970).

The next enzyme that is considered to be involved in starch synthesis, based on genetic evidence, is ADPG pyrophosphorylase which catalyzes the reaction

G-1-P + ATP --------> ADPG + PPi

Tsai and Nelson (1966) and Dickinson and Preiss (1969b) showed that the starch deficient mutant shrunken-2 (sh2), was deficient in ADPG pyrophosphorylase indicating that ADPG pyrophosphorylase is required for normal starch synthesis.

The G-1-P is thought to arise from the conversion of UDPG into G-1-P and UTP by UDPG pyrophosphorylase. There is no genetic evidence for the involvement of UDPG pyrophosphorylase but its involvement has been proposed by Tsai, Salamini and Nelson (1970) and Tsai (1974).

The activity of ADPG pyrophosphorylase coincides with the time of maximum starch synthesis in maize (Ozbun et al. 1973, Tsai, Salamini and Nelson 1970) which indicates an involvement in starch synthesis. The activity of ADPG pyrophosphorylase also is correlated with starch synthesis in potato (Hawker, Marschner and Krausa 1979, Sowokinos 1976, Marea and Marschner 1980), wheat (Moore and Turner 1969), pea (Turner 1969) and barley (Baxter and Duffus 1973). Recondo and Leloir (1961) found that ADPG, in comparison to UDPG, was the substrate for soluble starch synthetase suggesting that the production of ADPG was needed for starch synthesis. The only substrate for soluble starch synthetase in maize is ADPG (Hawker and Downton 1974).

ADPG pyrophosphorylase, an allosteric enzyme, is considered to be regulatory in the synthesis of starch in chloroplast and possibly in storage tissue (Preiss 1978), Ghosh and Preiss 1966, Kaiser and Hanham 1979, Sanwal and Preiss 1967, Sharma and Bhatia 1980). The intracellular localization of ADPG pyrophosphorylase is in the chloroplast of leaf tissue and algae (Mares, Hawker and Possingham 1978, Kombrink and Weber 1980).

An examination of ADPG pyrophosphorylase in tobacco, rice, sorghum, barley, tomato, beet and kidney bean revealed that all had similar allosteric properties (Sanwal et al. 1968). All were activated by 3-PGA and inhibited by inorganic phosphate The inhibition by Pi is reversible by 3-PGA. The activity of ADPG pyrophosphorylase in maize is also modulated by 3-PGA and Pi (Preiss, Lammel and Ebbraw 1971, Dickinson and Preiss 1969a).

The sh2 gene is considered to be the structural gene for ADPG pyrophosphorylase in maize (Hannah and Nelson 1975, 1976, Hannah, Tuschall & Mans 1980) based on dosage effect and enzyme stability. Recently, Tuschall and Hannah (in press) showed that revertant alleles of sh2 conditioned pyrophosphorylase with different allosteric properties.

The final reaction in the conversion of sucrose into starch is the transfer of glucose to a glucan primer by the action of starch synthetase:

ADPG + Primer(n) ——— Primer(n+1) + ADP

The reaction is thought to be carried out by two glucan synthesizing enzymes: soluble and starch granule bound starch synthetase. Nelson and Rines (1962) and Nelson and Tsai (1964) showed that the starch granule bound starch synthetase was lacking in the mutant waxy (wx). Similar results were obtained for waxy rice (Reum et al. 1970).

The wx mutation does not condition a loss of starch but rather an alteration in the normal starch composition. In wx, the level of amylopectin is increased whereas amylose is lacking. Without the bound starch synthetase wild type levels of starch are still maintained.

Soluble starch synthetase is specific for ADPG in maize (Ozbun, Hawker and Preiss 1971), soybean and pea (Fryman, Desouza and Cardini 1966). In potato (Hawker, Ozbun and Preiss 1972) and maize (Hawker and Downton 1974) soluble starch synthetase was reported to support unprimed glucan synthesis.

There is evidence that soluble starch synthetase may play the major role in starch synthesis. In soybean, Murata and Akazawa (1964) showed that [^{14}C] ADPG was transferred to starch more effectively than [^{14}C] UDPG. Soluble starch synthetase has been correlated with the time of rapid starch synthesis in rice (Perez et al. 1975)

Baxter and Duffus (1971) found that the activity of ADPG dependent starch synthetase was correlated with the time of rapid starch synthesis in barley. The isolation of the sh2 mutants, which are deficient in the production of ADPG also points to the role of soluble starch synthetase. This would be expected if glucose was added to starch through conversion of ADPG by soluble starch synthetase.

Based primarily on genetic evidence, as reviewed above, starch synthesis is thought to occur as presented in the upper portion of Figure 1. Yet for this pathway, only sucrose synthetase and ADPG pyrophosphorylase seem to be vital for starch synthesis, based on the isolation of genetic mutants. Starch deficient mutants that lack UDPG pyrophosphorylase or soluble starch synthetase had not been isolated. It is possible however, that mutants for UDPG pyrophosphorylase or soluble starch synthetase are lethal and thus cannot be isolated. However, all mutants that have been isolated exhibited a low level of enzyme activity suggesting that the complete absence of an enzyme activity is lethal. By the same logic, mutants of the other enzymes should exist.

Other pathways have been proposed which are based on physiological evidence. From the distribution of metabolites in isolated amyloplasts, Liu and Shannon (1981b) proposed that carbon flows through 3-PGA and DHAP as illustrated in the bottom portion of Figure 1.

A potential technique for examining starch synthesis is the use of *in vitro* tissue culture systems. Such systems would be open to manipulation and could provide information about starch synthesis. For tissue culture systems to be useful in metabolic examinations of starch synthesis, they must reflect *in vivo* metabolism. Tissue specific enzymes important to starch synthesis must be expressed.

Shannon and Batey (1973), reportedly established tissue cultures of maize endosperm for analysis of starch synthesis. Chu and Shannon (1975) found that supplementing the growth medium with sucrose led to starch accumulation in the endosperm cells. However, amylose content and total starch content of the cells were low in comparison to intact kernels. From these data they suggest that the cultured cells did not develop all of the enzymes necessary for normal starch synthesis.

Freeling, Woodman and Chang (1976) found differential expression of Adh in callus tissues derived from immature maize embryos. Again, this suggests that specific gene expression may not occur in callus or suspension tissue culture systems.

Recently, Gengenbach (1977) has succeeded in growing intact maize ovules to maturity in culture. Blocks of tissue, consisting of kernels and supportive tissue, were

removed from the ear and placed in culture where they would develop to maturity. Although the presence of specific enzymes was not determined, the cultured kernels metabolized labeled amino acids (Gengenbach, personal communication).

The technique of *in vitro* kernel development may be useful in examining starch synthesis in the maize endosperm as well as studies of seed development. While the kernel blocks are grown *in vitro*, the kernels are intact. This may maximize the tissue specific expression of enzymes in the endosperm tissue and thus, overcome the problems of gene expression found with callus and suspension tissue culture systems. More importantly, this system may offer a unique opportunity to study starch synthesis under controlled conditions.

For *in vitro* kernel development to be useful in the examination of starch synthesis and seed development it must reflect starch synthesis as it occurs *in vivo*. The primary objective of this investigation was to examine development of wild type, *sh1* and *sh2* kernels grown *in vitro* to determine if growth in culture mimics growth *in vivo*.

After determining that development in culture was comparable to growth *in vivo*, *in vitro* kernel development was utilized to investigate carbon metabolism in the maize

endosperm. Kernels were grown on different carbon sources to determine if kernels would develop in different carbon sources and if so, if growth in different carbon sources would affect starch synthesis and thus be useful for analysis of starch biosynthesis.

MATERIALS AND METHODS

Biological Materials

Wild type maize as well as the mutants shrunken-1 (sh1) and shrunken-2 (sh2) were analyzed. Normal maize was F2 progeny of WG4A X 182E. The shrunken-1 allele, 7205, having approximately 5% of the wild type level of sucrose synthetase for both the cleavage and synthetic reactions was provided by P. Chourey. The shrunken-2 material, having approximately 5% of the wild type level of ADPG pyrophorylase, was the commercial cultivar "Florida Stay Sweet". Plants were grown at the horticulture unit in Gainesville or in a greenhouse.

Development In Vitro

The first series of experiments was devoted to examining development of caryopsis (kernels) using kernel

culture methods. These observations were made in an effort to quantify development of caryopsis grown in culture.

In the initial experiments, ears were harvested 2dpp and placed in culture using the techniques developed by Gengenbach (1977) as described below. After 8 days in culture the kernel blocks were scored to determine the percentage fertilization which was based on kernel enlargement.

These blocks were visually scored at 5 day intervals for growth and development through 35dpp. Some of the kernels were removed from culture at 5 day intervals and examined with the aid of a dissecting microscope to determine their stage of development.

After 35dpp the remaining kernel blocks were removed and air dried for 1 week. These were then examined to determine developmental maturity and the total percentage of development as defined by the percentage of fertilized kernels that reached maturity. In this case, a kernel was considered to have reached maturity if it exhibited growth in culture and, upon drying, did not have any cracks or other external abnormalities.

Improvement of In Vitro Development

The next series of experiments were designed to increase the percentage of kernels that could be classified

as normal at the end of 30dpp. In the first experiment, the time that the kernel blocks were placed in culture after pollination was varied from 2dpp to 7dpp. Again W64A X 182E maize was used. After 30 days in culture they were removed and air dried for 1 week. As in the previous experiment the percentage of fertilization was based on kernel enlargement at 10dpp. After air drying the kernels were scored for development.

Other procedures were examined in an effort to improve development in culture. Results indicate that neither of the procedures that involved surgically removing some of the kernels was effective in increasing in vitro development. In one experiment the kernel blocks were placed in culture and allowed to develop until 10dpp. The petri dishes were then opened and every other developing kernel was sacrificed by piercing the kernel with a scalpel. The remainder of the kernels were then placed in culture and allowed to develop until 35dpp. Observations were made every 5 days until the seeds were mature. At this time the percent development was determined as described above.

Another experiment similar to the one above was performed in an effort to improve in vitro development. Ovules were placed in culture as described and allowed to develop until 10dpp. The kernel blocks were then removed

from culture and all but 1 of the developing ovules were removed for each kernel block with the aid of a scalpel. The kernel blocks were returned to culture and allowed to mature.

Kernel Culture Procedure

After harvest the ears were transported to the laboratory and held at 4 C. All ears were placed in culture within 4 hours of harvest, although it was determined that ears could be stored for 24 hr without damage. Immediately before placement in culture, the outer husks of the ears were removed and the inner husks were sprayed with 95% ethyl alcohol and flamed. All successive operations were carried out under aseptic conditions. The inner husks were removed and the ear was divided longitudinally into 3 or 4 segments. Blocks of kernels approximately 4 rows wide and 6 to 8 kernels long were removed from each segment with the aid of a scalpel. The outer 2 rows of kernels were removed from each segment and the cob tissue was trimmed to a flat base with tapered sides. The resultant kernel blocks consisted of 12 to 16 kernels supported on a 4 to 6 mm thick piece of cob tissue.

Culture Medium

Kernel blocks were placed in a Murashige and Skoog medium as modified by Gengenbach (1977) and as described here. Sugar concentrations were 150 g/l of sucrose, glucose or fructose. For the combination glucose and fructose treatment 75 g/l of each sugar was used. The medium was filter sterilized or sterile sugars were added after the medium, minus sugars, was autoclaved.

The medium was supplemented with one mg/l 2,4-dichlorophenoxyacetic acid and one g/l of casin hydrolysate. Ten milligrams/liter of streptomycin sulfate was added to control bacterial contamination. Two to 4 kernel blocks were placed in each 100X25mm petri dish containing 50 ml of culture medium. All cultures were grown at 26 C in darkness.

Germination Measurements of Kernels Grown in Culture

Kernels of the three genotypes were grown in culture with sucrose, glucose, fructose or glucose plus fructose as the carbon source. After 30 days in culture, kernels deemed to be mature were removed, air dried for at least 2 weeks, and were used to examine germination.

For determination of germination, the seeds were placed between sheets of moist blotting paper and germinated in the dark at 25 C for 1 week. Total germination was the percentage of seeds that had germinated after 1 week. Germination was defined as radical emergence.

Another parameter measured was the germination rate index which relates the percentage of germination to the germination rate by the following formula (Schmiell & Golberg 1971):

$$GRI = \frac{\sum_{N=1}^{N=C} X_n\ (N)}{X}$$

X_n = The number of seeds that germinated on day N.

N = Day that germination was measured on.

C = Total days of germination test.

X = Sum of all seeds that germinated during test.

Root and shoot lengths were also measured. Seedlings were removed after the 7 day germination test. Root length and shoot length were measured to the nearest half millimeter.

Development of Enzymatic Extraction Procedure

In an effort to maximize activity of sucrose synthetase, ADP-glucose pyrophosphorylase and starch synthetase field grown, normal maize was used in a series of extraction and assay procedures

Normal maize endosperms, 22dpp, were ground 1:1 (W:V) in a chilled mortar with either a 0.01M TRIS-maleate buffer (pH7.0), 0.1M potassium phosphate buffer (pH7.5) containing 0.01M EDTA and 0.005M GSH, or 0.03M potassium phosphate buffer (pH 7.0).

After grinding, the homogenate was centrifuged at 34,000g for 20 min. Aliquots of the supernatant were assayed for activity of the 3 enzymes using standard reaction mixtures.

In other experiments the supernatants were dialyzed overnight against either 0.01 TRIS-maleate buffer (pH7.0), 0.05M HEPES buffer (pH7.0) containing 0.005M DTT or 0.05M potassium phosphate(pH7.0). Activity of the 3 enzymes was determined after dialysis.

In other experiments the supernatants from the initial centrifugation were salt fractionated by addition of an equal volume of saturated ammonium sulfate (77gm/100ml). After storage at 4 C for 1 hr., the enzyme preparations

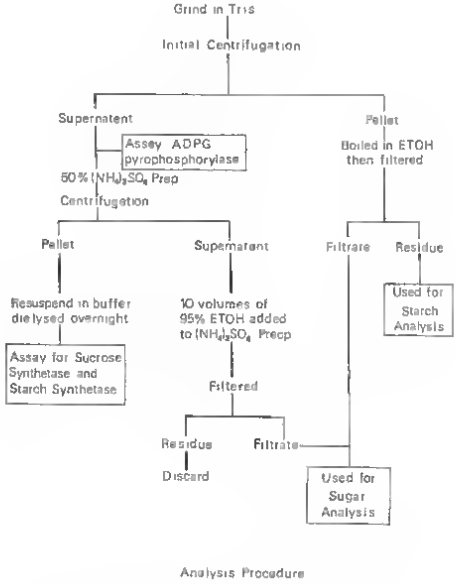
were centrifuged at 27,000g for 15 min. The pellet was resuspended in one-half the original volume in one of the 3 dialysis buffers and dialyzed overnight against that buffer. Aliquots were then used for activity measurements of the 3 enzymes. All combinations of buffers were examined to optimize the extraction procedure for maximum enzyme activity.

From these experiments it was determined that the optimum method was to extract in 0.01M TRIS-maleate buffer (pH 7.0). ADP-glucose pyrophosphorylase was assayed after the initial centrifugation. Sucrose synthetase and starch synthetase were assayed after salt fractionation and overnight dialysis against the 0.01M TRIS-maleate buffer.

Common Extraction Procedure

After determination of the optimum procedure for enzyme recovery, methods were developed to recover sugars and starch from the enzyme preparations. This extraction procedure is summarized in Figure 2.

Frozen kernels were removed from the cob tissue and the endosperms were seperated from the embryo and pericarp. The endosperms were ground 1:1 (W:V) in chilled 0.01M TRIS-maleate buffer (pH7.0). The homogenate was centrifuged at 34,000g for 25 min. The supernatant



Analysis Procedure

Figure 2- Flow chart for isolation of enzymes, sugars and starch from endosperm tissue.

contained the 3 enzymes as well as some of the soluble sugars. The pellet, containing the starch and some of the soluble sugars, was saved for later extraction. An aliquot of the crude supernatant was removed, diluted 1:5 with water and immediately used for determination of ADP-glucose pyrophosphorylase activity

To separate the soluble enzymes from the soluble sugars, an equal volume of saturated ammonium sulfate was added to the crude supernatant. After incubation in ice for 1 hr., the solution was centrifuged at 27,000g for 15 min. The pellet containing the enzymes was resuspended in one-half the original volume of the extraction buffer and dialyzed overnight against buffer. This partially purified enzyme preparation was used for assays of sucrose synthetase and starch synthetase activities.

To the supernatant from the salt fractionation, which contained the soluble sugars, 10 volumes of 95% ethyl alcohol was added to precipitate the ammonium sulfate. This was filtered through #4 Whatman filter paper and the filtrate, containing the soluble sugars saved. The residue was discarded.

The starch containing pellet from the initial centrifugation was boiled in 10 volumes of 95% ethyl alcohol for 30 min. After filtration through Whatman #1 filter paper the filtrate, which contained the ethyl

alcohol soluble sugars, was pooled with the other solable sugar fraction and used in the sugar analysis. The residue, which contained the starch, was dried in a 60 C oven and stored in a desicator until analyzed.

The validity of the common extraction procedure for accurate measurements of enzymes and sugars was examined by comparing results from the common extraction technique with results obtained by using optimized extraction methods for each component. Also, radioactive sugars were added to the initial extract and recovery of the sugars at each step of the extraction procedure was determined. Results from these comparisons support the validity of the common extraction method.

Enzyme Assays

a. Sucrose synthetase (UDP-glucose D-fructose 2 glucosyltransferase, E.C.2.4.1.13) was assayed by the method of Chourey and Nelson (1979). Ten microliters of the dialyzed, enzyme preparation were added to the sucrose synthetase reaction mixture which consisted of 60 µmoles of glycine-NaOH buffer (pH 9.0), 2 µmoles magnesium sulfate, 10 umoles of fructose and 2 µmoles of UDP-glucose in a total volume of 0.4 ml. Control tubes lacked UDP-glucose

After incubation at 37 C for 10 min. 0.1 ml of 10N NaOH was added to terminate the reaction. The reaction mixtures were then boiled in a water bath for 12 min to destroy the residual fructose.

Sucrose formed was measured using the Roe (1934) method by adding 2 ml of 0.1% alcoholic resorcinol and 6 ml of 30% HCl to the reaction mixtures. The tubes were placed in a water bath at 80 C for 8 min. The tubes were then removed and cooled to room temperature under a stream of water. Absorbance was measured at 540nm with a Beckman DU spectrophotometer.

To examine the reliability of the Roe method for sucrose determination, some samples of known sucrose concentration were analyzed by both the Roe method and gas-liquid chromatography. Results indicate that the Roe method gives an accurate indication of sucrose concentration.

b. ADP-glucose pyrophosphorylase (ATP:α-D-glucose 1-phosphate adenyltransferase, E.C. 2.7.7.27) was assayed by the formation of ADP-[^{14}C] glucose from [^{14}C] glucose-1-phosphate using a modification of the method of Dickinson and Preiss (1969) as described by Hannah, Tuschall and Mans (1980).

Ten microliters of the crude enzyme preparation were diluted 5 fold with distilled water immediately before

assaying. The reaction mixture consisted of 8.0 umoles of HEPES buffer (pH 8.0), 2 umoles of magnesium chloride, 50 ug of BSA (bovine serum albumen , 0.2 umoles ATP, 2 umoles 3-PGA, 0.2 umoles α-D-[^{14}C] glucose-1-phosphate, and 10 ul of the diluted crude enzyme preparation in a final volume of 50 ul. Controls lacked ATP.

After incubation for 15 min at 37 C the tubes were boiled in a water bath for 1 min to terminate the reaction. After cooling to room temperature 1.25 units of bacterial alkaline phosphatase was added to each tube to cleave the unreacted glucose-1-phosphate. The tubes were incubated for one hr at 37 C.

Twenty microliters of the reaction mixture was spotted on DEAE filter disks. After 3, 10 min washes in distilled water the disks were dried and monitored for radioactivity by scintillation spectrometry.

c Soluble starch synthetase (soluble ADPglucose-starch glucosyltransferase E.C.24.1.21 was assayed by incorporation of [^{14}C] glucose from ADP-[^{14}C] glucose (discussed below) into amylopectin by a modification of the methods of Ozbun et. al. (1971), Tsai, Salamini and Nelson (1970) and Hawker and Downton (1974). The reaction mixture consisted of 12 umoles HEPES buffer (pH 7.5), 0.5 umoles ADP-[^{14}C] glucose (46,000cpm), 0.5 umoles EDTA, 5 mg of amylopectin and 10 ml of the partially

purified enzyme preparation to a final volume of 100 µl. Controls lacked amylopectin. The reaction was carried out for 30 min at 37 C.

The reaction was terminated and the amylopectin solubilized by the addition of 1 ml of 0.1N NaOH. Methanol was added to a final concentration of 75%. The starch was pelleted by centrifugation at 34,000g and washed 3 times by NaOH solubilization followed by methanol precipitation and centrifugation. After the third wash the starch pellet was solubilized in 0.2 ml of 0.1N NaOH followed by adding 1 ml of methanol to suspend the starch.

The starch suspension was placed on glassine filter disk and washed with 10 ml of methanol. After drying the disk was monitored for radioactivity by scintillation spectrometry.

d. Invertase (α-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26) was assayed by the methods of Jaynes and Nelson (1971a). Endosperms were ground 1:1 (W:V) in 0.05 M TRIS-malaate buffer (pH 6.0) followed by centrifugation at 22,000g for 10 min. The supernatant was dialysed overnight against 2 L of 0.01 TRIS-malaate (pH 7.0) containing 1mM mercaptoethanol.

The reaction mixture contained 0.1 ml of 0.01 M sucrose, 0.01 ml of 0.3 M sodium acetate buffer (pH 5.0) and 1 to 10 µl of enzyme solution plus water to a final volume of 0.175 ml. Incubation times were 5 to 15 min.

To terminate the reaction, 0.6 ml of Nelson's reagent (1944) was added followed by heating at 95 C for 15 min. The mixture was cooled and 0.6 ml of arsanomolybdate reagent was added. Absorbance was read at 520nm

Preparation of [^{14}C] ADPG

ADP-[^{14}C] glucose was prepared by a modification of the methods of Preiss and Greenberg (1972). Four microcuries of a uniformly labelled [^{14}C] glucose-1-phosphate solution (239 mCi/mmole) were evaporated to dryness under a stream of air. One hundred microliters of the standard ADP-glucose pyrophosphorylase reaction mixture minus unlabeled glucose-1-phosphate were added. Twenty microliters of a highly purified preparation of ADP-glucose pyrophosphorylase, a gift from Dr. Glen Weiser, were added and the mixture was incubated at 37 C for 1 hr. The reaction was terminated by boiling for 1 min. followed by treatment with alkaline phosphatase as described above.

The reaction mixture was concentrated under a stream of air then streaked onto Whatman #1 paper. Authentic samples of ADP-glucose were spotted on both sides of the streaked reaction mixture. The mixture was chromatographed (descending) for 24 hr. in a solvent system consisting of

95% ethanol : 1M ammonium acetate (5:2) pH 7.5. After chromatography, the authentic ADP-glucose spots were located with a UV lamp and the area between the spots was removed. This strip was washed in 500 ml of absolute ethanol for 5 hours followed by drying. The ADP-[^{14}C] glucose was eluted from the paper with distilled water.

Starch Determination

The dried starch residue isolated after treatment with hot ethyl alcohol was ground to a fine powder. One hundred milligrams of the powder was solubilized in 10 ml of 0.5N NaOH, followed by addition of 70 ml of distilled water. The pH was adjusted to 4.5 with acetic acid and the volume adjusted to 100 ml. Ten milligrams of amyloglucosidase were added to 1 ml of the solution followed by incubation at 55 C for 1 hr. Starch was then measured as free glucose using a Yellow Springs Instrument model 27 Glucose Oxidase Analyzer.

For measurement of starch in dried seeds the seeds were ground in 5 volumes of 95% ethyl alcohol. After filtering through Whatman #4 paper the residue was dried. One hundred milligrams were then used for measurement of starch as described above.

Uptake of Radiolabeled Sugars into Whole Kernels

Kernel blocks of normal, sh1 and sh2 were placed in culture as previously described. At 20dpp, the kernel blocks were removed and divided to produce pairs of kernels with the cob tissue still attached to the kernel base.

The developing kernels were transferred into fresh culture medium containing [^{14}C] sugar (Amersham). The [^{14}C] sugar (glucose or sucrose, 1 uCi/kernel, specific activity = 253 mCi/mMole and 497 mCi/mMole respectively) was located in depressions made in the agar medium. Kernels were supplied with the labeled sugar on which they were grown. For determination of sucrose hydrolysis, kernels grown on glucose were supplied with labeled sucrose.

After a 1 hr pulse in the [^{14}C] sugar, the kernels were transferred back into the original medium for the chase period. Samples were taken immediately after removal from the [^{14}C] sugar and at hour intervals through 4 hr. The embryo and pericarp were removed and the endosperms were frozen until analyzed.

The endosperms were ground 1:1 (w:v) in 95% ethyl alcohol followed by centrifugation at 34,000g for 20 min. The supernatant was concentrated under a stream of air and lyophilized. Four hundred microliters of ethyl alcohol

were added to each sample and 50 µl of the solution was spotted on Whetman #1 paper. Chromatography (descending) was carried out in a solvent system consisting of butanol acetic acid : water (3:1:1) for 64 hr. The chromatographs were cut into 1 cm squares, placed in scintillation fluid and monitored for radioactivity by scintillation spectrometry.

Gas-Liquid Chromatography

Sugars were quantified by gas-liquid chromatography using a modification of the methods of Ferguson, Dickinson and Rhodes (1979). Two milliliters of the soluble sugar extract were placed in a vial and dried in an oven at 60 C. Lipids were removed from the samples by addition of 1 ml of hexane and 1 ml of water for lipid-sugar partitioning. The samples were mixed and allowed to stand until the layers separated. The hexane phase, containing the lipids was removed and the aqueous phase dried in an oven at 60 C. Analysis of this technique revealed that no sugars were lost into the hexane phase during partitioning.

Oximes of the dried sugars were made by addition of 0.1 ml of STOX Oxime-Internal Standard Reagent (Pierce Chemical Co.) which contained 25 mg/ml of hydroxylamine hydrochloride and 6 µg/ml of phenyl-β-α-glucopyranoside in

pyridine. The vials were incubated for 30 min. at 70 C then cooled to room temperature before silylation.

The oxime-sugars were silylated by the addition of 0.1 ml of N-trimethylsilylimidozole followed by vortexing the sample for 30 sec. The vials were incubated for 30 min. at room temperature before injection. One microliter of the silylated sugars was injected into a Hewlett Packard 5710A gas chromatograph programed at 150 C for 2 min. followed by a linear rise in temperature to 250 C at a rate of 6 C/min. Injection port temperature was 300 C and the detector temperature was 350 C. The column was stainless steel (6' X 1/8") packed with 3% OV-17 on Chromosorb W(HP) 80/100 mesh (Altech). The carrier gas was Hellum at 40 ml/min.

Ion-Exchange Chromatography

Ion-exchange chromatography was performed on the labeled nucleotide sugars isolated by descending paper chromatography in an attempt to identify the sugars. The methods of Randerath and Randerath (1964) were used. The papers containing the nucleotide sugars were removed from the scintillation fluid, dipped twice in toluene and dried. The nucleotide sugars were eluted with water and concentrated to a volume of approximately 50 µl. The nucleotide sugars were applied to the P.E.I. plates and

developed as above. After ion-exchange chromatography the areas corresponding to the nucleotide sugars were removed and radioactivity was determined by scintillation spectrometry.

Hydrolysis of [^{14}C] Labeled Sucrose

The [^{14}C] labeled sucrose isolated by descending paper chromatography was hydrolyzed with invertase (Sigma). The papers corresponding to the labeled sucrose were removed from the scintillation fluid, dipped twice in toluene and air dried. The sucrose was eluted from the paper with water and lyophilized.

One milliliter of an acetate buffer (pH 4.5) containing 200 units of invertase was added to the lyophilized sugar and incubated for 2 hr. at 55 C. The reaction mixture was lyophilized and then dissolved in 100 ul of 95% ethyl alcohol and subjected to descending paper chromatography as described previously. The areas corresponding to sucrose, glucose and fructose were removed and monitored for radioactivity.

Development In Vitro

In the first experiment, blocks of wild type kernels were placed in culture at 2dpp. After 10 days in culture the percentage of fertilization was inferred from the percentage of kernel enlargement. Some kernels were then allowed to develop to maturity while others were removed at 5 day intervals and examined with the aid of a dissecting microscope.

Approximately 80% of the kernels appeared to be fertilized based on kernel enlargement at 10dpp (Figure 3). The ovules that had not enlarged were necrotic or were quite small and exhibited no further signs of development.

Approximately 25% of the kernels that were fertilized developed to maturity and contained both embryo and endosperm (Figure 4). An additional 10% exhibited some growth but growth was terminated before 30 days in culture

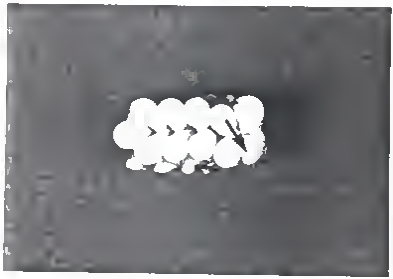


Figure 3. Block of wild type kernels at 10dpp. Most of the kernels have enlarged indicating fertilization. Caryopses that were not fertilized (arrows) exhibit no growth

Figure 4. Block of sh2 kernels at maturity (35dpp). The normal kernels are large and have started to shrink due to loss of water. The atypical kernels (arrows) are smaller and appear more shrunken

(Figure 4). These kernels were distinguishable from normal kernels by there small size due to depressed development (Figure 4).

The atypical kernels included those that had embryos but little endosperm and kernels that had no discernible embryo but did possess a poorly developed but identifiable endosperm. In some instances the endosperms of these seeds were larger than normal but were filled with fluid and, when opened and examined with a dissecting microscope, did not appear to be cellular. The majority of the kernels that exhibited ovule enlargement but did not develop to maturity exhibited arrested development before 15dpp. Most became necrotic and by 20dpp were collapsed and extremely small. The normal kernels would germinate and develop normal coleorhiza and coleoptiles when placed under standard germination conditions whereas atypical kernels failed to germinate although they did imbibe water.

Improvement of In Vitro Development

After the initial analysis of development in culture, experiments were performed to improve the percentage of kernels that could be classified as normal after 30 days in culture. In the first experiment the time between pollination and harvest was increased. Results of this

experiment (Figure 5) revealed that increasing the time between pollination and harvest increased the number of kernels that were normal. While there was little increase in the number of atypical kernels, the number of normal kernels tripled.

From the data collected, it would appear that the best time to place the fertilized ovules in culture is 5dpp on (Figure 5). Ovule enlargement had occurred at 5dpp making it possible to determine which of the caryopses had been fertilized. This was an important factor when considering the number of kernel blocks needed for the experiments. It was difficult to distinguish between fertilized and unfertilized ovules at 2dpp and 3dpp.

Also, 73% of the kernels that were fertilized were normal after 30 days in culture (Figure 5). Even though there was a slight increase in development from 5dpp to 7dpp the older kernels were not used because of the difficulty in dissecting the kernel blocks from the cob tissue.

Development of Kernels Grown in Culture

Wild type kernels and sh1 and sh2 kernels were placed in culture at 5dpp in media containing sucrose as the carbon source. Kernels were removed at 5 day intervals,

sliced through the center, and examined with a dissecting microscope.

When the caryopses were placed in culture, the endosperms were small and the nucellar tissue comprised the bulk of the tissue. The embryo at this time could not be found with a dissecting microscope although it was possible to tease out the endosperm tissue from the nucellar tissue.

At 10dpp the endosperm had displaced most of the nucellar tissue. At this time it was possible to remove part of the endosperm which contained the embryo and examine it with a compound microscope. The embryo was in the late globular stage in which the plumule-radicle axis had been established. In some instances the symmetry of the embryos could be established with the presence of plumule and radicle initials and early stages of scutellum formation.

The endosperm, at 15dpp, had almost totally displaced the nucellar tissue and the cells of the endosperm were noticeably larger than at 10dpp. The embryo at 15dpp had a definite shape and the plumule and radicle were well established. The scutellum was also apparent.

Between 20dpp and 25dpp there was little change in the endosperm cells except for some cell enlargement and the obvious presence of starch granules. The embryos had

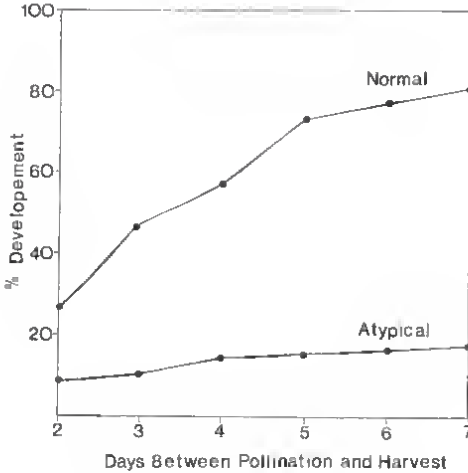


Figure 5. Effect of varying days between harvest and placing kernels in culture on kernel development.

undergoes the most radical changes with the appearance of leaf primodia and the appearance of the coleorhize. The size of the embryo increased from 15dpp to 25dp.

At 30dpp the kernels began to lose moisture as was evident by the formation of a dent in the crown of the kernel. The endosperm was hard and the cells were filled with starch. There were several seedling leaves present in the embryo and a well defined coleorhize and radicle.

Through the initial stages of growth, development of the mutants was similar to that in normal. Differences became apparent around 20-25dpp and were only associated with the endosperms. Development of the embryos was similar in all 3 genotypes.

The sh1 and sh2 endosperms contained less starch at 25dpp in comparison to wild type. The endosperms of sh2 were filled with fluid and starch grains were not obvious at 25dpp. There was starch present at 25dpp in sh1.

By 30dpp sh1 and sh2 kernels had lost moisture. The sh2 endosperms were beginning to collapse and there was little evidence of starch accumulation. The sh1 endosperms, at 30dpp, had not collapsed and starch was obvious.

There exist considerable differences between the sh1 and sh2 kernels at maturity. The sh2 endosperms had collapsed and there were obvious spaces between endosperm

tissue and embryo. Some starch was present in the sh2 kernels but it was low very in comparison to wild type and sh1. The sh1 kernels had a considerable amount of starch present at maturity though the level was much less than in normal. The starch was located in the periphery of the kernel and there was a cavity in the interior of the endosperm tissue.

Growth of Kernels On Several Carbon Sources

Kernels of all 3 genotypes were placed in culture at 5dpp with sucrose, glucose, fructose or glucose plus fructose as the carbon source. At 35dpp the kernels were removed and weighed to determine if seed weight would be affected by growth on the different sugars. The weights of the kernels are given in Table 1. Wild type kernels had the greatest average seed weight followed by sh1 and sh2 seeds.

Seeds of wild type maize grown in the presence of the different sugars had wild type phenotypes at maturity (Figure 6). All kernels appeared to have a large amount of starch, based on visual observations. The kernels possessed a dented crown which is characteristic of the wild type phenotype. Kernels that were grown with sucrose as the carbon source had a significantly greater weight

Figure 6. Wild type kernels grown on different sugars and in the field. From left to right, F=field grown, S=kernels grown on sucrose, G=glucose, F=fructose, GF=glucose + fructose.

than those seeds grown with any of the other carbon sources indicating that sucrose was the superior carbon source (Table 1).

Seeds of sh2 grown in culture in the presence of the different carbon sources had their expected phenotype at maturity (Figure 7). Seeds of sh2 grown on sucrose had a significantly greater weight than kernels grown on the other sugars indicating that, as in wild type, sucrose was the superior carbon source (Table 1).

At maturity, seeds of sh1 grown in the different carbon sources had their characteristic phenotype in that they exhibited a concave crown (Figure 8). However, differences in denting of the crown were observed and it was possible to visually separate seeds grown on sucrose and glucose. Nevertheless, all seeds had concave crowns and thus were classified as sh1. Unlike wild type and sh2 kernels, sh1 kernels grown on glucose had a significantly greater weight than seeds grown on the other carbon sources (Table 1). Reducing sugars appear to be a better carbon source for sh1 in comparison to sucrose.

Germination and Growth of Kernels Grown in Culture

After weighing, seeds of the 3 genotypes were analyzed for germination rate, as defined by the germination rate

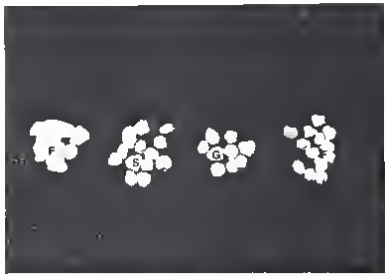


Figure 7. Kernels of sh2 grown on different sugars and in the field. From left to right, F=field grown, S=kernels grown on sucrose, G=glucoee, F=fructose, GF=glucose + fructoee

Figure 8. Kernels of *sh1* grown on different sugars and in the field. From left to right, F=field grown, S=kernels grown on sucrose, G=glucose, F=fructose, GF=glucose + fructose.

Table 1. Weight of kernels grown on different sugar sources. Kernels were grown to maturity in culture, air dried, then weighed. Approximately 100 kernels of each genotype were weighed.

Phenotype	Sugar Source	Dry Weight (g/kernel)
Wild Type	Sucrose	.135a[z]
	Glucose	.157b
	Fructose	.126b
	Glu+Fru	.139b
shrunken-1	Sucrose	.093b
	Glucose	.125a
	Fructose	.107b
	Glu+Fru	.113b
shrunken-2	Sucrose	.088a
	Glucose	.068b
	Fructose	.051b
	Glu+Fru	.059b

[z] Seperation by Duncan's Multiple Range Test

index, and for total percentage of germination. The lengths of roots and shoots were also determined.

The germination parameters for wild type seeds are given in Table 2. Kernels grown on sucrose had a greater germination rate index (Table 2) and a greater total germination percentage than kernels grown on the other sugars. The next highest germination rate index and total germination were for seeds grown on glucose followed by seeds grown on glucose plus fructose. The lowest germination rate index and total germination were for kernels grown on fructose. Root and shoot lengths were similar for kernels grown in all carbon sources (Table 2).

The germination parameters for sh2 kernels are given in Table 2. As in wild type, sh2 kernels grown on sucrose had a greater germination rate index and percentage germination than did kernels grown on reducing sugars. The next greatest germination rate index and total germination were for kernels grown on glucose followed by kernels grown on glucose plus fructose and then fructose. The root and shoot lengths were similar for kernels grown in all sugars.

Germination parameters for sh1 are given in Table 2. In contrast to normal and sh2, kernels grown on glucose as the carbon source had the highest total germination percentage and the greatest germination rate index. Following kernels grown on glucose, the next highest

Table 2 Growth Measurements of Seeds Grown to Maturity in Culture.

Genotype	Sugar Source	Total Germination	GRI[z]	Root[x] Length	Shoot[x] Length
Wild Type	Sucrose	96±3.2	4.11	18.7±4.2	10.6±1.3
	Glucose	78±2.8	3.10	15.4±3.9	9.3±3
	Fructose	75±4.2	2.76	12.3±3.4	8.9±2.3
	Glu+Fru	77±5.2	2.82	13.8±2.3	9.2±1.9
ahrunken-1	Sucrose	52±3.8	2.69	15.4±2.1	7.2±2.1
	Glucose	76±3.6	2.83	16.2±3.4	9.6±1.8
	Fructose	63±2.2	2.63	12.8±1.5	7.8±1.8
	Glu+Fru	60±3.6	2.78	14.0±3.2	7.8±1.5
shrunken-2	Sucrose	43±3.8	2.58	12.2±3.1	8.2±1.9
	Glucose	36±4.3	2.51	12.1±3.1	9.6±1.1
	Fructose	34±2.9	2.46	12.4±2.9	9.2±0.9
	Glu+Fru	32±3.7	2.42	11.9±1.4	9.2±1.4

[z] Germination Rate Index
[x] All measurements in centimeters

germination indices were for kernels grown on glucose plus fructose, then fructose. Kernels grown on sucrose had the lowest germination rate and the least germination rate index. As in wild type and sh2 there were no differences in shoot or root lengths of kernels grown on the different carbon sources.

Starch Content of Kernels Grown to Maturity in Culture

Analysis of germination indicated an association between seed germination and seed weight. In order to determine if seed weight was correlated with starch content, kernels of all 3 genotypes grown to maturity on the different sugars were evaluated for starch content. Results are given in Table 3. The same pattern for seed weight and germination measurements was also found for starch content.

Wild type kernels had the greatest starch content of the 3 genotypes examined (Table 3). Kernels of wild type grown on sucrose had a higher starch content than did kernels grown on reducing sugars (Table 3). The next highest starch content was found for kernels grown on glucose followed by glucose plus fructose, then fructose.

Kernels of sh2 had the least amount of starch of the 3 genotypes (Table 3). Kernels of sh2 grown on sucrose had a

Table 3. Starch Content of Mature Kernels Grown in Culture.

Genotype	Sugar Source	%Starch[z]
Wild Type	Sucrose	81±3.3
	Glucose	68±3.6
	Fructose	60±2.8
	Glu+Fru	64±3.5
shrunken-1	Sucrose	40±3.9
	Glucose	60±3.4
	Fructose	44±2.8
	Glu+Fru	40±4.5
shrunken-2	Sucrose	32±1.6
	Glucose	25±3.0
	Fructose	22±1.9
	Glu+Fru	28±2.3

[z]Percent of Dry Weight

higher starch content then did kernels grown on the other sugars (Table 3). Kernels grown on glucose had the next highest starch content followed by kernels grown on glucose plus fructose then fructose.

As in seed weight and germination, shi kernels grown on reducing sugars had a greater starch content than kernels grown on sucrose. Kernels grown on glucose had the greatest starch content (Table 3). This was followed by kernels grown on the other reducing sugars. Kernels grown on sucrose had the lowest starch content

Enzymes in Developing Kernels Grown in Different Carbon Sources

The activity of sucrose synthetase, ADPG pyrophosphorylase and starch synthetase were measured throughout development in kernels of the 3 genotypes grown on sucrose, glucose or fructose. Enzyme activity was examined in order to determine if the enzymes were present in kernels developed in culture, and if so, whether the sugar source effected enzymatic levels Futhermore, this work would help determine if growth in culture mimics growth *in vivo*. It was also of interest to determine if differences in enzyme activity could be correlated with the differences in seed weight found when kernels were grown on the different sugars.

In the mutants, enzymatic activity of the deficient enzyme was examined only at 20dpp and 25dpp (Table 4). The level of sucrose synthetase in sh1 was approximately 5% of the activity found in wild type or sh2 endosperms produced in vitro (Table 4). This is similar to the results obtained by Chourey and Nelson (1976) and Chourey (1979) for sucrose synthetase activity in sh1, grown in vivo. Activity of sucrose synthetase was similar in sh1 kernels grown on sucrose, glucose or fructose.

The activity of ADPG pyrophosphorylase in sh2 was approximately 3% of the activity in wild type and sh1 endosperms (Table 4); in agreement with results obtained by Hannah and Nelson (1975,1976) for sh2, grown in vivo. Activity was similar in kernels grown on sucrose, glucose or fructose

The specific activity of sucrose synthetase in wild type and sh2 kernels grown on the different carbon sources is given in Figures 9 and 10. In general, the specific activities were similar in wild type and sh2 kernels. No activity was found at 10dpp in either genotype. In general, the maximum activity occurred at 25 to 30dpp. By 35dpp the activity had declined considerably.

The activity of ADPG pyrophosphorylase in wild type and sh1 kernels that were grown on the different sugars are given in Figures 11 and 12. Both genotypes had similar

Table 4. Percentage of wild type levels of sucrose synthetase in sh1 and activity of ADPG pyrophosphorylase in sh2 kernels at 20 and 25dpp.

Genotype	Enzyme Measured	Activity[z] 20dpp	25dpp
sh1	sucrose synthetase	5.7%	5.21%
sh2	ADPG pyrophosphorylase	3.31%	3.54%

[z]Percentage of wild type levels of enzymes at 20 and 25dpp.

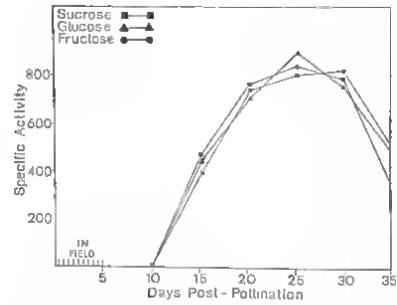


Figure 9. Specific Activity of sucrose synthetase throughout development in endosperms of wild type kernels grown on sucrose, glucose or fructose.

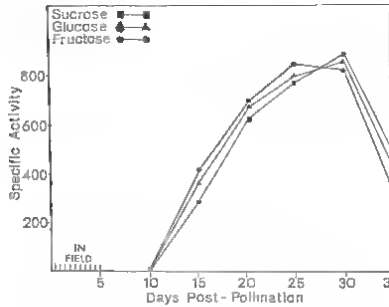


Figure 10. Specific Activity of sucrose synthetase throughout development in endosperms of sh2 kernels grown on sucrose, glucose or fructose.

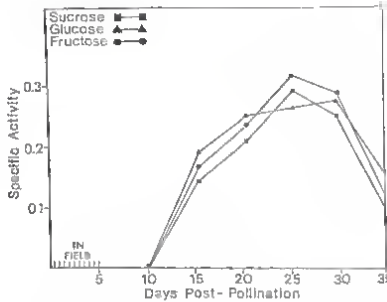


Figure 11. Specific Activity of ADPG pyrophosphorylase throughout development in endosperms of wild type kernels grown on sucrose, glucose or fructose.

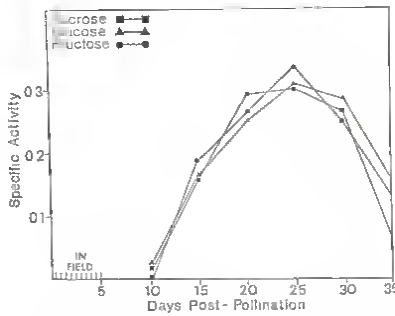


Figure 12. Specific Activity of ADPG pyrophosphorylase throughout development in endosperms of sh1 kernels grown on sucrose, glucose or fructose.

specific activities and activity profiles throughout development. Also, there were no differences in activity related to carbon source.

Activity of starch synthetase in wild type, *sh1* and *sh2* kernels grown on sucrose, glucose or fructose are given in Figures 13, 14 and 15. Activity of starch synthetase was similar in all 3 genotypes. Enzyme profiles were similar with maximum activity at 25 to 30dpp.

Analysis of Sugars by Gas Chromatography

The levels of sucrose, glucose and fructose were measured throughout development in kernels of the 3 genotypes grown on the different sugars. This was done to determine if growth *in vitro* was a reflection of what occurs *in vivo* and if there was an accumulation of sugars in the different mutants. Mutant phenotypes and the build up of sugars in the mutants grown in culture would be expected if growth *in vitro* mimics *in vivo* growth.

It was possible to resolve the silyated sugar derivatives of sucrose, glucose and fructose by the use of gas chromatography. The retention times of the derivatives of sucrose, glucose and fructose were approximately 11.3 min., 4.6 min. and 4.0 min. respectively.

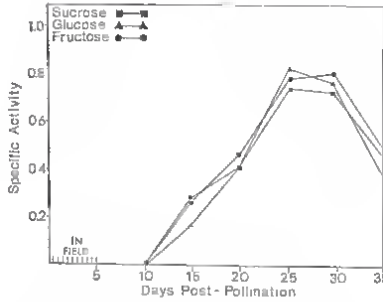


Figure 13. Specific Activity of starch synthetase throughout development in endosperms of wild type kernels grown on sucrose, glucose or fructose.

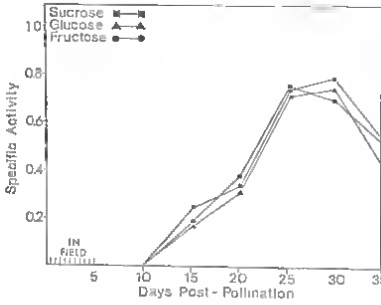


Figure 14. Specific Activity of starch synthetase throughout development in endosperms of sh1 kernels grown on sucrose, glucose or fructose.

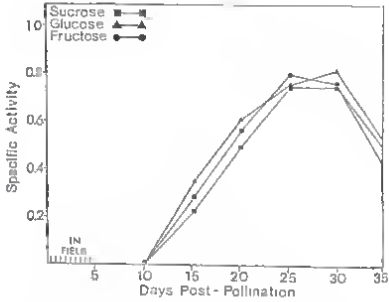


Figure 15. Specific Activity of starch synthetase throughout development in endosperms of sh2 kernels grown on sucrose, glucose or fructose

The concentrations of sugars in normal, ah1 and sh2 grown on sucrose are given in Figures 16, 17 and 18. The sucrose level in wild type remained low throughout development (Figure 16) and peaked at 20-25dpp. Levels of fructose and glucose in wild type increased gradually through 25dpp and declined thereafter (Figure 16). The concentration of sucrose was greater than the combined concentration of fructose and glucose from 20dpp to maturity.

The concentration of sucrose in ah1 increased approximately threefold between 15dpp and 20dpp (Figure 17), and declined thereafter. The concentration of sucrose in ah1 from 15dpp to 35dpp was approximately 3 times greater than that found in normal. As in normal, there was little change in the concentrations of fructose or glucose through development (Figure 17). At later times examined, the concentration of sucrose in ah1 was greater than the concentration of fructose plus glucose. In general, the concentrations of glucose and fructose were greater in ah1 than in normal.

The concentration of sucrose in ah2 was greater than that in either of the other genotypes (Figure 18). Sucrose increased threefold from 15dpp to 20dpp and continued to increase to 30dpp. There was a slight decline in concentration from 30 to 35dpp. The concentrations of

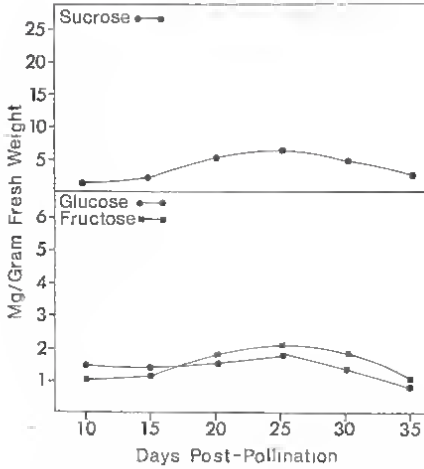


Figure 16. Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of wild type grown on sucrose.

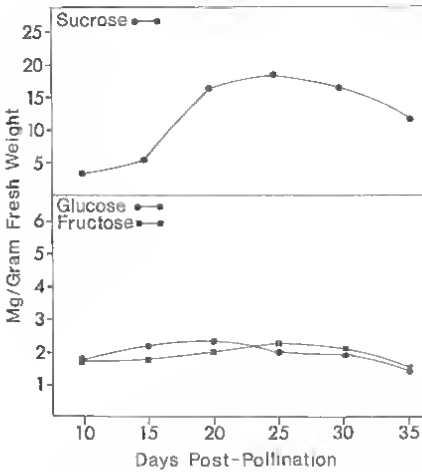


Figure 17 Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of sh1 grown on sucrose.

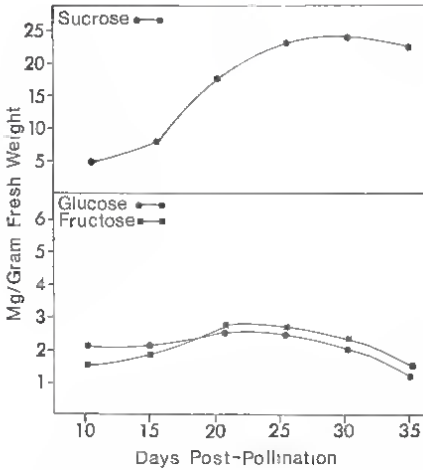


Figure 18. Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of sh2 grown on sucrose.

fructose and glucose increased from 15 to 20dpp and declined through 35dpp (Figure 18) The concentration of sucrose was greater at all time intervals than the concentration of glucose plus fructose.

The changes in sugar concentrations in the wild type and sh2 genotypes grown on sucrose are similar to results obtained by Creech (1968), of kernels grown in vivo, in that there was an increase in the amount of sugars in sh2 in comparison to wild type. The concentrations of sugars at 25dpp in sh1 are also in agreement with results obtained by Chourey & Nelson (1976).

The concentrations of sugars in kernels grown on glucose or fructose are given in Figures 19 and 20 for normal, 21 and 22 for sh1 and 23 and 24 for sh2.

When glucose was supplied, the level of glucose was approximately 2 to 3 times greater than that observed when sucrose was the carbon source (Figures 19,21,23). However, the concentration of fructose in kernels grown on glucose was reduced compared to those grown on sucrose. Kernels grown on glucose also had less sucrose than kernels grown on sucrose. The concentration of sucrose for all genotypes was approximately 50% to 90% the level found in kernels grown on sucrose from 20 to 35dpp. Interestingly, there was a buildup of sucrose in sh1 and sh2 similar to the buildup observed when sucrose was the carbon source.

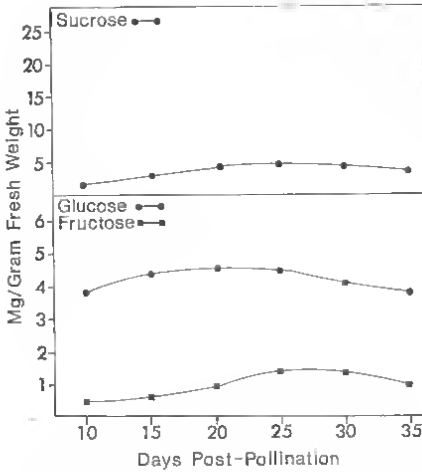


Figure 19. Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of wild type grown on glucose.

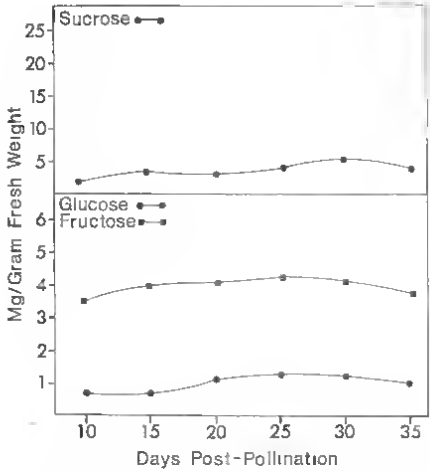


Figure 20. Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of wild type grown on fructose.

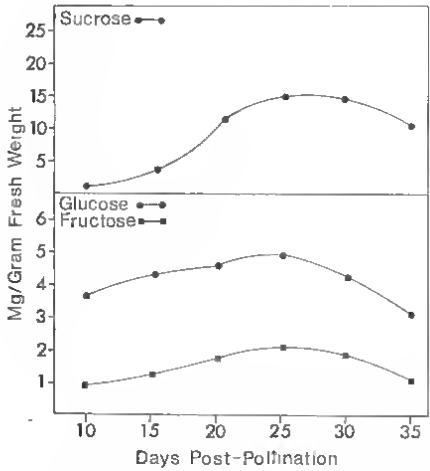


Figure 21. Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of *sh1* grown on glucose.

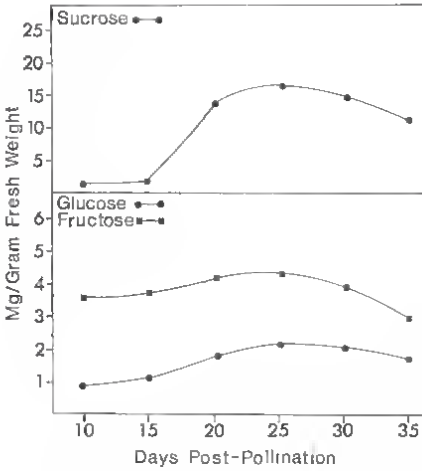


Figure 22. Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of *eh1* grown on fructose.

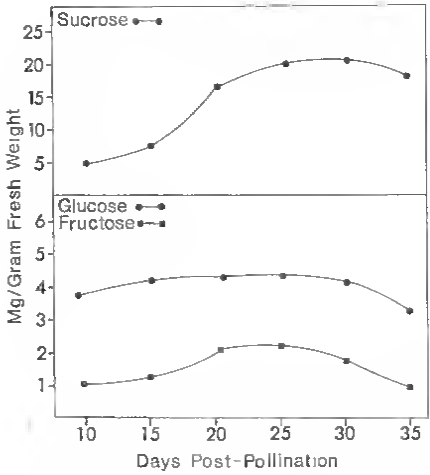


Figure 23. Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of eh2 grown on glucose.

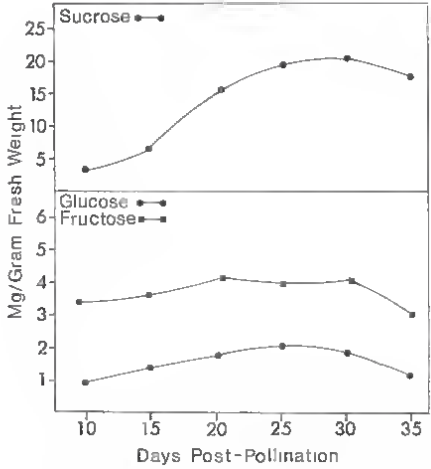


Figure 24. Concentrations, in mg/g fresh weight, of sucrose, fructose and glucose in endosperms throughout development in kernels of sh2 grown on fructose.

Supplying fructose increased the amount of fructose approximately 2 times and reduced the concentration of glucose by half during the early times examined whereas the concentration of glucose was equivalent to that found in kernels grown on sucrose in the later time intervals (Figures 20,22,24). As is the case of kernels grown on glucose, kernels grown on fructose had 10% to 20% less sucrose than when grown in sucrose.

Starch Content In Developing Kernels Grown in Different Carbon Sources.

The amount of starch in developing normal, sh1 and sh2 kernels grown in the presence of the different carbon sources was examined. Data from kernels grown on sucrose were used in analysis of in vitro development. These data were useful in determining how growth in culture mimics growth and development in the field.

Determination of starch content in developing kernels grown in the different carbon sources was done to determine if differences in carbon source would affect the production of starch in the endosperms. Previous results showed that starch content in mature kernels was altered when grown in the presence of the different carbon sources. Also, seed weight and germination parameters were altered. Analysis of developing kernels would determine when differences in starch became apparent during development.

Starch content of wild type kernels grown on the different sugars are given in Figure 25. There is little difference in the starch content at 10dpp and 15dpp of kernels grown in any of the carbon sources. From 20dpp to 35dpp, kernels grown on sucrose had more starch than kernels grown on reducing sugars. In agreement with previous data, kernels at 35dpp grown on sucrose had the greatest starch content followed by kernels grown on glucose and then kernels grown on fructose. Starch content in wild type kernels grown on all carbon sources was greater than the starch content of the other genotypes.

Starch levels of sh1 kernels grown on the different sugars are given in Figure 26. As is normal, the starch contents are similar for all 3 carbon sources through 15dpp. From 20dpp to 35dpp kernels grown on glucose had the greatest starch level. At 35dpp kernels grown on glucose had the greatest starch level followed by kernels grown on fructose and then kernels grown on sucrose.

The starch levels of sh2 kernels grown on the different sugars are given in Figure 27. As in the other genotypes there was little difference in starch content through 15dpp. From 20dpp to 35dpp, kernels grown on sucrose had the greatest starch level though there was little difference in the amount of starch between all 3 sugar sources. The next greatest starch level was found in

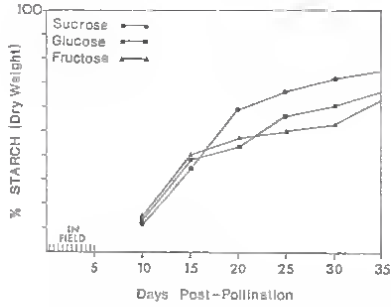


Figure 25. Starch content, in percent dry weight, of endosperms of wild type kernels grown on sucrose, glucose or fructose.

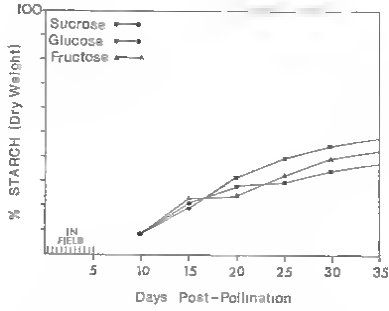


Figure 26. Starch content, in percent dry weight, of endosperms of shl kernels grown on sucrose, glucose or fructose.

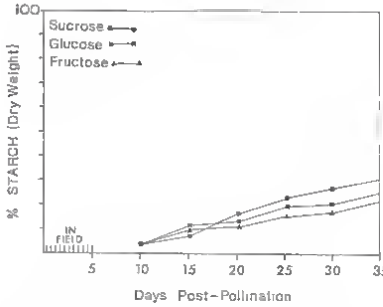


Figure 27 Starch content, in percent dry weight, of endosperms of sh2 kernels grown on sucrose, glucose or fructose.

kernels grown on glucose followed by kernels grown on fructose. The amount of starch in sh2 kernels was less than in the other genotypes.

Uptake of [^{14}C] Labeled Sucrose or Glucose Into Kernels

[^{14}C] labeled sucrose or glucose was supplied to 20dpp kernels of wild type, sh1 and sh2 grown in vitro, to determine the initial distribution of label in the endosperm tissues. Specifically, it was of interest to determine if sucrose was transported into the endosperms tissue or if it was hydrolyzed before entry into the endosperm tissue. These experiments were also used to determine the rate of uptake and conversion of the sugars.

Results obtained above (Figures 16-18) indicated that kernels grown on sucrose had greater sucrose in the endosperm tissue than did kernels grown on reducing sugars. Also, kernels grown on reducing sugars had increased levels of the sugar on which they were grown (Figures 19-24). By the use of radiolabeled sugars it is possible to determine the rate of transport of sugars into the endosperm tissue as well as metabolic conversions that may occur.

Analysis of extracts of kernels by descending paper chromatography revealed the presence of labeled compounds that co-chromatographed with sucrose, glucose and fructose

Labeled compounds also were found that chromatographed with nucleotide sugars as well as phosphorylated hexoses. Other labeled compounds were also observed but were not identified. Representative profiles of monitored chromatographs of all 3 genotypes are given in Figures 28-33.

Approximately 55% of the label was found in sucrose at the earliest time period examined (Figures 34-36) in all 3 genotypes when the kernels were fed sucrose. Recovery of labeled sucrose in the endosperm tissue does not necessary show that sucrose is transported into the endosperm tissue. Since, as shown above, glucose can be used to synthesize sucrose in the endosperm tissue, labeled sucrose could have been broken down before transport into the endosperm tissue and then resynthesized.

In an effort to examine whether sucrose is broken down during transport into the endosperm, kernels that were grown with glucose as the carbon source were supplied with labeled sucrose. After a 1 hr. pulse, the endosperms were extracted and subjected to paper chromatography. The sucrose peak was isolated and hydrolyzed with invertase. The resultant mixture was rechromatographed and the areas corresponding to glucose and fructose were removed and counted.

Figure 2#. Representative profils of [^{14}C] labeled compounds saparated by deacendlng paper chromatography over a fi$_{1}$e hour period from wild type kernels aftar axposnra to [^{14}C]-sucrose. The aucrose peak la at 22 cm, the glucose peak ia at 32 cm and the fructose pesk ia at 38 cm. Sucrose, glucose and fructose accounted for the majority of the label recovared. Addltlonal peaks were found at 1 cm which corresponda to nucleotide sugara and at 7 cm which corresponda to phoaphorylated haxoses.

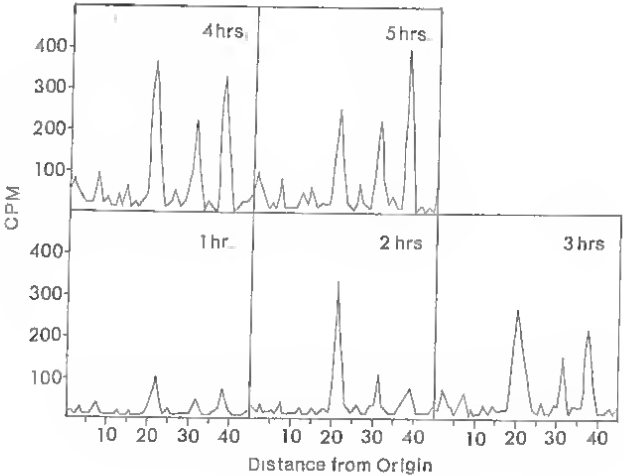

4 hrs.
5 hrs.
1 hr.
2 hrs
3 hrs
CPM
400
300
200
100
10 20 30 40
Distance from Origin

Figure 29. Representative profile of [^{14}C] labeled compounds seperated by descending paper chromatography over a five hour period from wild type kernels after exposure to [^{14}C]-glucose. The sucrose peak is at 22 cm, the glucose peak is at 32 cm and the fructose peak is at 38 cm. Sucrose, glucose and fructose accounted for the majority of the label recovered. Additional peaks were found at 1 cm which corresponds to nucleotide sugars and at 7 cm which corresponds to phosphorylated hexoses.

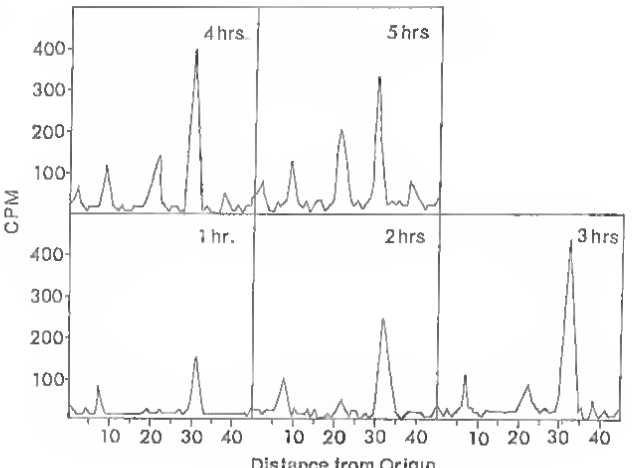

4 hrs.
5 hrs
1 hr.
2 hrs
3 hrs
CPM
400
300
200
100
10 20 30 40
Distance from Origin

Figure 30. Representative profile of [^{14}C] labeled compounds seperated by descending paper chromatography over a five hour period from *shl* kernels after exposure to [^{14}C]-sucrose. The sucrose peak is at 22 cm, the glucose peak is at 32 cm and the fructose peak is at 38 cm. Sucrose, glucose and fructose accounted for the majority of the label recovered Additional peaks were found at 1 cm which corresponds to nucleotide sugars and at 7 cm which corresponds to phosphorylated hexoses.

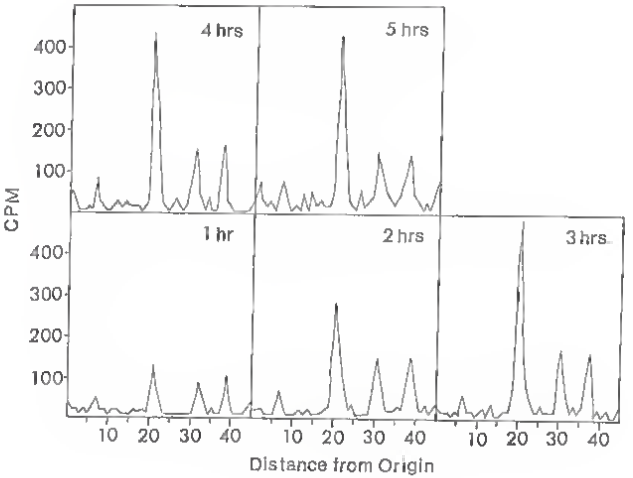

4 hrs
5 hrs
1 hr
2 hrs
3 hrs
CPM
400
300
200
100
10 20 30 40
Distance from Origin

Figure 31. Representative profile of [^{14}C] labeled compounds separated by descending paper chromatography over a five hour period from sh2 kernels after exposure to [^{14}C]-glucose. The sucrose peak is at 22 cm, the glucose peak is at 32 cm and the fructose peak is at 38 cm. Sucrose, glucose and fructose accounted for the majority of the label recovered. Additional peaks were found at 1 cm which corresponds to nucleotide sugars and at 7 cm which corresponds to phosphorylated hexoses.

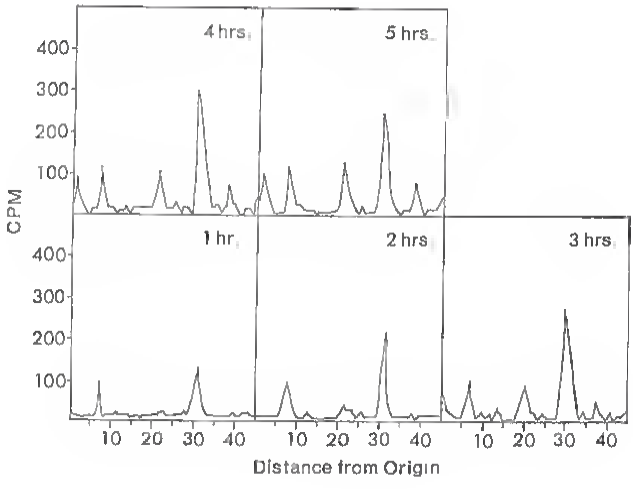

4 hrs.
5 hrs.
1 hr.
2 hrs.
3 hrs.
CPM
400
300
200
100
10 20 30 40
Distance from Origin

Figure 32. Representative profile of [^{14}C] labeled compounds separated by descending paper chromatography over a five hour period from sh2 kernels after exposure to [^{14}C]-sucrose. The sucrose peak is at 22 cm, the glucose peak is at 32 cm and the fructose peak is at 38 cm. Sucrose, glucose and fructose accounted for the majority of the label recovered. Additional peaks were found at 1 cm which corresponds to nucleotide sugars and at 7 cm which corresponds to phosphorylated hexoses.

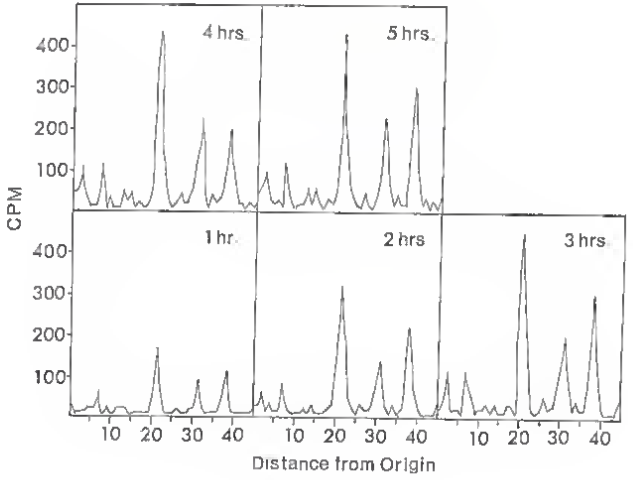
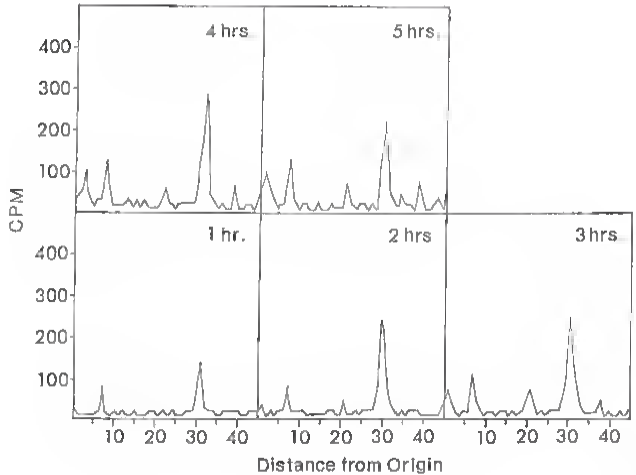

4 hrs.
5 hrs.
1 hr.
2 hrs
3 hrs.
CPM
400
300
200
100
10 20 30 40
Distance from Origin

Figure 33. Representative profile of [^{14}C] labeled compounds separated by descending paper chromatography over a five hour period from *sh2* kernels after exposure to [^{14}C]-glucose. The sucrose peak is at 22 cm, the glucose peak is at 32 cm and the fructose peak is at 38 cm. Sucrose, glucose and fructose accounted for the majority of the label recovered. Additional peaks were found at 1 cm which corresponds to nucleotide sugars and at 7 cm which corresponds to phosphorylated hexoses.


4 hrs.
5 hrs.
1 hr.
2 hrs
3 hrs.
CPM
400
300
200
100
10 20 30 40
Distance from Origin

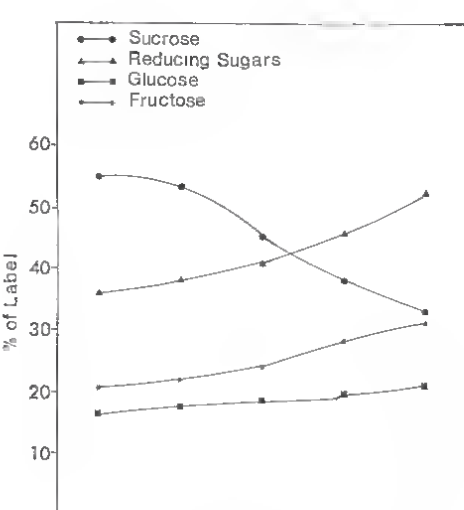


Figure 34. Percentage of ethanol soluble, [^{14}C] labeled sucrose, glucose, fructose and total reducing sugars extracted from endosperms over a 5 hour period after exposure of wild type kernels to [^{14}C] sucrose. Kernels were exposed to the labeled sugar for 1 hr. The start of exposure to the label is -1 hour on graph and removal from the label is at time 0. The chase period was 4 hours.

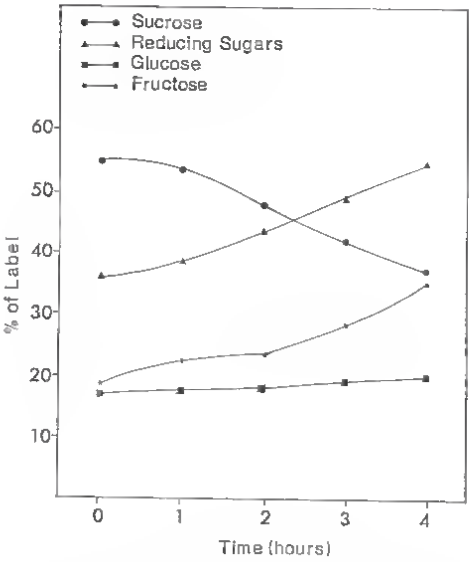


Figure 35. Percentage of ethanol soluble, [^{14}C] labeled sucrose, glucose, fructose and total reducing sugars extracted from endosperms over a five hour period after exposure of ah2 kernels to [^{14}C] sucrose. Kernels were exposed to the labeled sugar for 1 hr. The start of exposure to the label is -1 hour on graph and removal from the label is at time 0. The chase period was 4 hours.

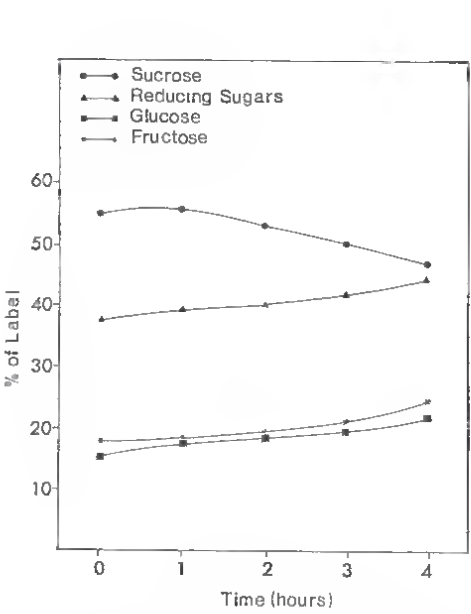


Figure 36. Percentage of ethanol soluble, [^{14}C] labeled sucrose, glucose, fructose and total reducing sugars extracted from endosperms over a five hour period after exposure of sh1 kernels to [^{14}C] sucrose. Kernels were exposed to the labeled sugar for 1 hr. The start of exposure to the label is -1 hour on graph and removal from the label is at time 0. The chase period was 4 hours.

The ratio of glucose to fructose of the supplied labeled sucrose was 1.08 and the ratio of glucose to fructose of the samples extracted from the endosperm tissue after a 1 hr. exposure to the labeled sucrose was 1.04. If sucrose was hydrolyzed one would expect an alteration in the ratio of the labeled hexose moieties. Hydrolysis and resynthesis of sucrose would lower the ratio, since the kernels were grown in glucose, and thus resynthesized sucrose would contain greater amounts of cold glucose than fructose. These results indicate that at a minimum 55% of the supplied sucrose is transported into the endosperm tissues without hydrolysis.

The amounts of total soluble label found in the endosperm tissue for 5 hr. after exposure to the labeled sugar are given in Figure 37. The profiles are similar for wild type and sh2 although the uptake was greater in wild type. From 0 hr., the time at which the kernels were removed from the labeled sugar, until 1 hr. there is an increase in label found in the tissue. This increase would be expected if the time needed for transport of sugar, from the cob tissue into the endosperm tissue, is greater than 1 hr. From the third through the fifth hour the amount of label declined which may indicate the movement of label into insoluble compounds or loss as CO_2.

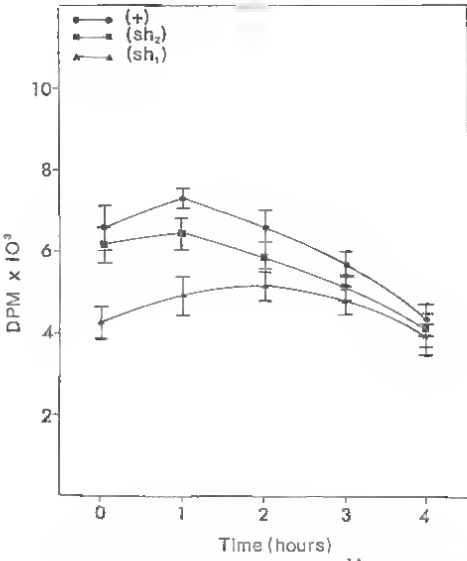


Figure 37. Total uptake in DPM of [^{14}C] sucrose into kernels of wild type, sh1 and sh2 over five hours. Time is after removal from the label.

The ratio of glucose to fructose of the supplied labeled sucrose was 1.08 and the ratio of glucose to fructose of the samples extracted from the endosperm tissue after a 1 hr exposure to the labeled sucrose was 1.04. If sucrose was hydrolyzed one would expect an alteration in the ratio of the labeled hexose moieties. Hydrolysis and resynthesis of sucrose would lower the ratio, since the kernels were grown in glucose, and thus resynthesized sucrose would contain greater amounts of cold glucose than fructose. These results indicate that at a minimum 55% of the supplied sucrose is transported into the endosperm tissues without hydrolysis.

The amounts of total soluble label found in the endosperm tissue for 5 hr after exposure to the labeled sugar are given in Figure 37. The profiles are similar for wild type and sh2 although the uptake was greater in wild type. From 0 hr., the time at which the kernels were removed from the labeled sugar, until 1 hr. there is an increase in label found in the tissue. This increase would be expected if the time needed for transport of sugar, from the cob tissue into the endosperm tissue, is greater than 1 hr. From the third through the fifth hour the amount of label declined which may indicate the movement of label into insoluble compounds or loss as CO_2.

In sh1, the uptake of label was the least of the 3 genotypes and the profile is different from that found in wild type or sh2 (Figure 37). The amount of label increased through 3 hr. whereas the peak occurred at 2 hr. in wild type and sh2. Unlike wild type and sh2, the amount of label in sh1 declined only slightly from the third to the fifth hour indicating less conversion in sh1 than in the other genotypes.

The relative amounts of sucrose, glucose and fructose through 5 hr. in wild type are given in Figure 34. For all time periods examined [^{14}C] labeled glucose, fructose and sucrose were found. Since label was found in all 3 sugars considerable conversion of sucrose into fructose and glucose occurred. The percentage of label in sucrose declined over time.

The relative amount of total label in reducing sugars increased with chase time and after 3 hr. there was more label in the reducing sugars than in sucrose. The amount of fructose in relation to the amount of glucose increased with time.

The relative amounts of sucrose, glucose and fructose in sh2 as a function of time are given in Figure 35. Results were similar to those obtained for wild type. As in wild type, label was found in sucrose, glucose and fructose at all times examined. The amount of label in

sucrose decreased through the 5 hr. period though not as much as in wild type.

Labeled reducing sugars increased through the 5 hr period and was greater than the label in sucrose by the fourth hour. As in wild type, the relative amount of label in fructose increased in relation to glucose through the 5 hr. period.

For wild type and sh2 the results indicate that sucrose as well as reducing sugars are found in the endosperm tissue. They indicate that 45% of the sucrose is hydrolyzed either before entry, or in the basal area, of the endosperm tissue. The relative increase in fructose in relation to glucose may indicate the involvement of sucrose synthetase in the breakdown of sucrose. Since fructose, and not glucose, is produced by sucrose synthetase one may expect that more fructose than glucose would be produced if sucrose synthetase does indeed break down sucrose.

The relative amount of label found in sh1 endosperms are given in Figure 36. As in wild type and sh2, labeled sucrose, glucose and fructose were found at all time periods examined. In contrast to wild type and sh2, the amount of label found in sucrose was greater than the amount of label in reducing sugars at all time intervals examined. There was an increase in the relative amount of label in sucrose through the first 3 hr. From the third

through fifth hour the amount of label in sucrose decreased as the amount of label in reducing sugars increased.

In contrast to wild type and sh2, fructose did not increase in comparison to glucose in sh1 endosperms. The absence of the increase in fructose may be due to the absence of sucrose synthetase. Without sucrose synthetase there is no production of fructose relative to glucose as in wild type and sh2. The equal amounts of glucose and fructose may indicate that sucrose is broken down by invertase. Sucrose breakdown is probably accomplished by invertases present in cells of the basal areas of the endosperm.

The different rate of sucrose breakdown observed in the 3 genotypes could possibly be attributed to lower invertase activity in sh1 thus leading to less sucrose breakdown. Invertase was measured in the 3 genotypes grown in vitro and in sh2 and wild type grown in vivo (Table 5). Invertase activities in field grown and culture grown kernels were the same.

The uptake of labeled glucose into kernels of wild type, sh1 and sh2 are given in Figure 38. The relative amounts of sugars found through the period examined in wild type, sh1 and sh2 are given in Figures 39, 40, and 41. Unlike the metabolism and uptake of sucrose the metabolism of glucose was similar in all 3 genotypes and will be discussed together.

Table 5. Invertase Activity in Field Grown and *In Vitro* Grown Maize Endosperms.

Genotype	*In Vitro* Grown	Field Grown
Wild Type	0.287±0.037[z]	0.286±0.021
shrunken-2	0.316±0.040	0.308±0.022
shrunken-1	0.228±0.021	y

[z] All values are specific Activity
[y] Not Examined

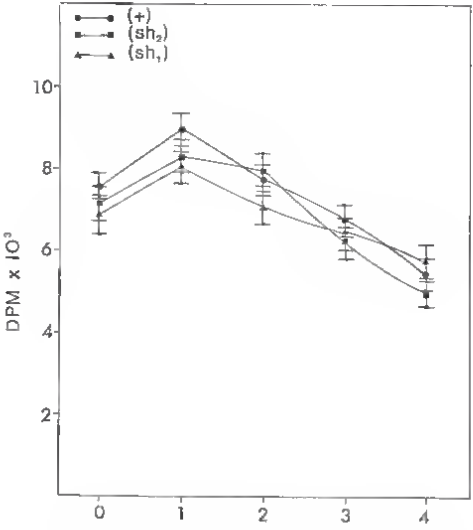


Figure 38. Total uptake in DPM of [^{14}C] glucose into kernels of wild type, sh1 and sh2 over five hours. Time is after removal from the label.

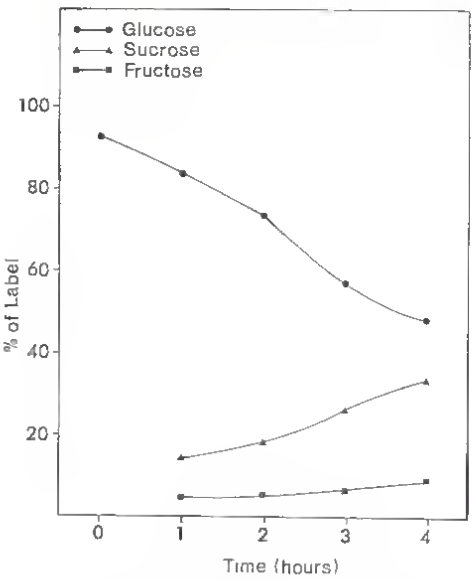


Figure 39. Percentage of ethanol soluble, [^{14}C] labeled sucrose, glucose and fructose extracted from endosperms over a five hour period after exposure of wild type kernels to [^{14}C] glucose. Kernels were exposed to the labeled sugar for 1 hr. The start of exposure to the label is -1 hour on graph and removal from the label is at time 0. The chase period was 4 hours.

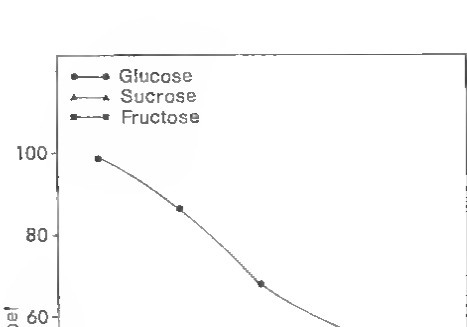


Figure 40. Percentage of ethanol soluble, [^{14}C] labeled sucrose, glucose and fructose extracted from endosperms over a five hour period after exposure of sh2 kernels to [^{14}C] glucose. Kernels were exposed to the labeled sugar for 1 hr. The start of exposure to the label is -1 hour on graph and removal from the label is at time 0. The chase period was 4 hours.

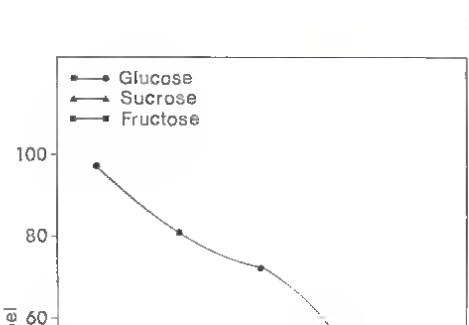


Figure 41. Percentage of ethanol soluble, [^{14}C] labeled sucrose, glucose and fructose extracted from endosperms over a five hour period after exposure of sh1 kernels to [^{14}C] glucose. Kernels were exposed to the labeled sugar for 1 hr. The start of exposure to the label is -1 hour on graph and removal from the label is at time 0. The chase period was 4 hours

At the end of the first hour only glucose was labeled. From the second to the fifth hour label was found in sucrose. The amount of label in sucrose increased through the 5 hr. examined. Also, there was label in fructose that increased through the fifth hour.

These results indicate that the uptake and metabolism of glucose is similar in all 3 genotypes. There appears to be considerable conversion of glucose into sucrose within 5 hr. By the fifth hour 30% of the label was found in sucrose.

Analysis of Nucleotide Sugars by Ion-Exchange Chromatography

Attempts were made to further characterize the nucleotide sugars that were labeled during exposure to the labeled sucrose. The areas corresponding to the nucleotide sugars were removed and applied to P.E.I.-Cellulose TLC plates. After development the areas that corresponded to ADPG and UDPG were removed an monitored for radioactivity. Radioactivity was found but the amount of label was too low to be useful for determining the identity of the nucleotide sugars.

In a second attempt to determine the identity of the radiolabeled compounds, the entire strip of the plate was removed and 1 cm pieces monitored for radioactivity. As in

the first experiment, radiation was detected but it was too low to be quantified.

The inability to isolate the nucleotide sugars could be due to the low specific activity of the labeled precursors supplied. Also, the nucleotide sugars may not be totally removed by the extraction method employed.

DISCUSSION

Development in Vitro

In the initial experiments, kernels of wild type, *sh1* and *sh2* were grown in culture with sucrose as the carbon source in order to determine if growth in culture mimics growth *in vivo*. Results obtained from these experiments suggest that growth of kernels in culture is similar to growth *in vivo*.

Firstly, the levels of the enzymes sucrose synthetase, ADPG pyrophosphorylase and starch synthetase as a function of development were characteristic of kernels grown *in vivo* (Figures 9-15). All enzymes were present in wild type kernels (Figures 9,11,13) and the enzyme associated with each mutation was found to be deficient in the respective genotypes. Developmental profiles of enzymatic activities were similar to results obtained by Tsai, Salamini and Nelson (1971), suggesting that the enzymes associated with starch biosynthesis are expressed in culture as *in vivo*.

Secondly, sugar levels during development in the 3 genotypes were similar to those that occur in vivo (Figures 16-18). There is an increase in the level of sucrose, in sh1 and sh2, similar to that found in field grown maize. Also, the increase in sucrose is greater in sh2 than in sh1 which is characteristic of these genotypes grown in the field (Figures 17 & 18) (Creech 1965, 1968 and Chourey & Nelson 1976).

Thirdly, the mature kernels of each genotype exhibited their distinct phenotypes (Figures 6-8). Wild type seeds were plump whereas the mutants were shrunken. The sh1 kernels had a characteristic concave crown whereas sh2 endosperms were quite collapsed.

Fourthly, the relative differences in starch content among the 3 genotypes grown in culture were similar to those found in seeds grown in vivo (Table 3) (Creech 1965, Chourey & Nelson 1976). Finally, the relative differences in germination among the the genotypes were similar in cultured and in vivo grown seeds (Hannah & Cantliffe 1976, Neas & Crane 1970) (Table 2).

These data indicate that growth in culture of wild type, sh1 and sh2 kernels is representative of growth in the field. The parameters examined in developing kernels, mature kernels and germinating kernels grown in culture were similar to field grown kernels.

Examination of Kernels Grown in Culture On Different Carbon Sources

Mature viable seeds can be produced on media containing sugars other that sucrose. Kernels of all 3 genotypes are capable of synthesizing sucrose as well as carrying out other sugar interconversions. Regardless of the carbon source, all 3 of the neutral sugars were present in the endosperm tissue at all times examined indicating that the endosperm tissues carry out sucrose synthesis and are capable of sugar interconversions (Figures 16-24).

Kernels grown on glucose contained 50 to 90% of the amount of fructose in comparison to that observed when kernels were grown on sucrose (Figures 19,21,23). Likewise, kernels grown on fructose contained 50 to 100% of the amount of glucose in sucrose grown kernels (Figures 20,22,24). The presence of the hexose, not supplied in the medium, could arise from the direct conversion of the supplied hexose by the action of mutases isomerases, kinases and phosphatases. Equally probable, the breakdown of sucrose, synthesized from the supplied hexose, could account for the presence of both hexoses.

The level of sucrose, in kernels grown on reducing sugars, is 50% to 90% of the level found in kernels grown on sucrose indicating that considerable sucrose is produced

in the endosperm tissues. There is a build up of sucrose in sh1 and sh2 kernels grown on hexose similar to the increase found in kernels grown on sucrose or kernels grown in the field. It is interesting that sucrose increases, and not reducing sugars, in the mutant kernels grown on reducing sugars. The build up of sucrose in kernels grown on reducing sugars may indicate an integral role of sucrose in starch biosynthesis. Shannon (1978) has suggested that sucrose is synthesized in the amyloplast and could be used for starch biosynthesis Conversely, the buildup of sucrose may reflect the storage of excess carbon that is not needed for starch biosyethesis in the metants.

These data also indicate that wild type levels of sucrose synthetase are not vital for sucrose synthesis. Sh1 kernels are greatly deficient in sucrose synthetase, nevertheless, they accumulate sucrose when grown on hexose or sucrose. The residual sucrose synthetase activity present in the sh1 kernels may account for the sucrose found or some other enzyme may be operating here.

Although kernels of the 3 genotypes were viable when grown on the different sugars, quantitative differences were observed during development and in the mature seeds. One of the most interesting observations is that starch content varied with carbon source in kernels of the same genotype. This provides a method of examining the

relationship between starch content and seed germination not possible before. It has long been considered that starch content and seed vigor were related based on comparisons made of several genotypes that differ in starch content. Yet, since different genotypes were compared it is not possible to rule out other factors. By comparing germination and growth of seeds of the same genotype, differing in the amount of starch they contain, the relationship between vigor and starch content may be futher elucidated.

It has been considered that starch content is an important energy source during germination since genotypes that have low starch content have poorer seed vigor than do genotypes with higher amounts of starch (Nass & Crane 1970, Hannah & Cantliffe 1976, Styer, Cantliffe & Hannah 1980). However, examination of starch deficient genotypes revealed that the differences in energy utilization were not sufficient to account for the differences in seed vigor (Styer, Cantliffe & Hannah 1980, Wann 1980). Recently, it has been shown that differences exist in the vigor of embryos of high and low starch genotypes suggesting that starch content of the endosperm may not be the only factor involved in seed vigor (Cantliffe & Styer 1981, Wann 1980).

There is a correlation between starch content, seed weight, germination rate index and total germination in all

3 genotypes examined. In general, seeds with the highest starch content, regardless of genotype, also had better germination. Germination differences do not appear to be related to the specific deficiencies found in the mutant genotypes but rather are related to starch content per se. The differences found in seeds of the same genotype grown on different sugars supports the suggestion that starch content per se plays a role in seed germination and thus seed vigor.

Transport of Sucrose Into The Maize Endosperm

In experiments examining the uptake of labeled sucrose, the first area of interest was to examine the transport of sucrose from the medium into the endosperm tissue. Results obtained by Shannon and Dougherty (1972) suggest that sucrose is hydrolyzed to glucose and fructose in the basal region of the kernel before transport into the endosperm tissue. This was based on the findings that reducing sugars accounted for greater than 80% of the radioactivity whereas sucrose accounted for less than 20% of the radioactivity at earlier time intervals in kernels from plants exposed to [^{14}C] CO_2 (Shannon 1968). The concentration of reducing sugars was found to be greatest in the placento-chalazal region (Shannon 1972) suggesting

that hydrolysis occurred in this region. It was also found that activity of invertase was greater, during active starch synthesis, in the basal regions in comparison to the other regions of the kernel, supporting the idea of sucrose hydrolysis in this region (Shannon and Dougherty, 1972). From an analysis of kernel anatomy and data obtained from microautoradiography, Shannon proposed that sucrose was transported through the symplast in the pedicle region and was hydrolyzed in the placento-chalazal and endosperm transfer cell region (Felker and Shannon 1980, Shannon 1978).

The results reported here indicate that sucrose can be transported into the endosperm tissue without hydrolysis. The experiments of Figures 34-36 indicate that at least 55% of the sucrose carbon transported into the endosperm entered as sucrose without hydrolysis. Kernels, grown with glucose as the carbon source, were supplied with labeled sucrose for 1 hr. followed by isolation of the sugars from the endosperm tissue. After 1 hr., 55% of the radioactivity was found in sucrose. The sucrose was isolated, hydrolyzed, and the ratio of glucose to fructose determined. If sucrose had been hydrolyzed and resynthesized, one would expect a change in the ratio of glucose to fructose because the concentration of glucose is greater than the concentration of fructose in kernels grown

on glucose. Therefore, if sucrose is hydrolyzed, the labeled glucose moiety would be diluted to a greater extent than would the labeled fructose moiety. Sucrose resynthesized from this pool would contain a greater concentration of labeled fructose than glucose.

In the results reported above the ratio of [^{14}C] glucose to [^{14}C] fructose of sucrose isolated from endosperm tissue was identical to that of the the labeled sucrose supplied to the developing seeds. Since the radiolabeled sucrose has equal amounts of label in the fructose and glucose moieties it is suggested that at least 55% of the supplied sucrose is transported into the endosperm cells without hydrolysis.

This is in contrast to the results obtained by Shannon (1968) which suggest that most of the sucrose is hydrolyzed before entry into the endosperm tissues. Results obtained by Jenner (1973, 1974), using techniques similar to those employed here, suggest that sucrose is not hydrolyzed during uptake into the developing wheat seed. In an investigation of sucrose uptake into wheat kernels, utilizing asymmetrically labeled sucrose in an in vitro system, Jenner determined that sucrose hydrolysis was not a prerequisite for transport into endosperm tissue (Jenner 1973, 1974). By the use of similar labeling techniques the transport of sucrose into potato slices (Hardy & Norton

1968), the phloem of beet (Giaquinta 1977), castor bean cotyledons (Kriedemann & Beevers 1967), bean pod tissue (Sacher 1966) and into carrot callus cultures (Edelman & Hanson 1971) is accomplished without hydrolysis.

The results reported above do not totally exclude a prerequisite for sucrose hydrolysis before entry into the endosperm tissue. First, if invertase, which is thought to be responsible for sucrose hydrolysis, has lowered activity in kernels grown in culture in comparison to kernels grown in the field then one might expect sucrose to enter the endosperm tissue without hydrolysis in kernel grown in vitro. This possibility was examined and it was found that invertase levels in field grown and in vitro grown wild type and sh2 maize were similar (Table 5) which suggest that hydrolysis of sucrose occurs to the same degree in culture and field grown maize. Also the level of invertase was similar in all 3 of the genotypes grown in culture (Table 5).

Secondly, if hydrolysis of sucrose occurs in a localized region at the kernel base, as suggested by Shannon (1978), then isolated pools of labeled hexoses might develop. If resynthesis of sucrose occurred in this region, it is conceivable for the newly resynthesized sucrose to contain equivalent amounts of labeled glucose and fructose moieties. This would agree with the results

reported here. However, in all probability an isolated pool of labeled hexoses does not occur due to the presence of the unlabeled glucose in the medium which is transported at the same time as the labeled sucrose.

Metabolism of Labeled Sucrose and Glucose

A series of pulse-chase experiments were conducted to investigate the metabolism of labeled sucrose and glucose in the endosperms of the 3 genotypes. These experiments were performed in order to determine the rate at which sugars are transported into the endosperm as well as to determine the initial transformations that sugars undergo once they reach the endosperm tissues. Also, these experiments might elucidate the role of sucrose synthetase in starch biosynthesis.

As in the analysis of mature seeds, several results from the pulse-chase experiments indicate that sucrose metabolism differs in sh1 in comparison to that in wild type and sh2 kernels. Furthermore, this difference can be attributed to the role of sucrose synthetase in the breakdown of sucrose and in starch synthesis.

First, the breakdown of sucrose occurred at a lower rate in sh1 in comparison to sh2 or wild type suggesting that sucrose is not as efficiently metabolized in sh1

(Figures 34-36). This would be expected if sucrose synthetase, which is greatly reduced in sh1, is involved in sucrose breakdown.

Secondly, there is a greater increase of reducing sugars in wild type and sh2 than in sh1 kernels supplied with [^{14}C] labeled sucrose (Figures 34-36). Again this suggests that sucrose is metabolized at a faster rate in sh2 and wild type than in sh1. More importantly, the amount of fructose produced from sucrose is greater than the amount of glucose in wild type and sh2 endosperms whereas the amounts of glucose and fructose are equivalent in sh1 endosperms. The increase in fructose relative to glucose observed in wild type and sh2 kernels would be expected if sucrose synthetase is involved in sucrose breakdown since fructose, and not glucose, is produced when sucrose is broken down by sucrose synthetase. The absence of an increase in fructose in sh1 adds credence to this possibility.

Thirdly, an observation that suggests the involvement of sucrose synthetase in sucrose breakdown and regulation of sucrose uptake is the difference found in the uptake of labeled sucrose between sh1 and the other genotypes (Figure 37). The uptake of labeled sucrose into the endosperm tissue was greater in wild type and sh2 than in sh1 (Figure 37) suggesting that the 5% of wild type levels of sucrose

synthetase in sh1 may affect the rate at which sucrose is transported into the endosperm cells. This would be expected if the net movement of sucrose, transported from the kernel base into the endosperm cells, is dependant on the ability of the endosperm cells to remove sucrose from the transported pool. A greater amount of sucrose could be transported into wild type and sh2 kernels because sucrose is removed from the transported pool by the action of sucrose synthetase. This then suggests the involvement of sucrose synthetase in the regulation of sucrose uptake into endosperm cells.

The analysis of the uptake and conversion of [^{14}C] labeled glucose also supports the notion that sucrose synthetase is involved in sucrose catabolism as well as providing additional evidence that sucrose can be synthesized in the maize endosperm. It was found that the uptake and metabolism of glucose is the same in all 3 genotypes. There were no differences in the amount of glucose found in the endosperm tissue (Figure 38), or in the conversion of glucose to sucrose in the 3 genotypes (Figures 39-41). This suggests that glucose metabolism is similar in all 3 genotypes. This also indicates that the alteration of sucrose metabolism in sh1 is not due simply to a general depression of sugar metabolism but rather is a specific response to sucrose. If this were not the case

then the metabolism of glucose in sh1 should be altered in the same fashion as is sucrose metabolism.

In all 3 genotypes there was conversion of labeled glucose into sucrose (Figure 39-41) which agrees with results obtained by Shannon (1972) that sucrose is resynthesized in the endosperm cells. Also, these results suggest that sucrose synthetase may not be involved in sucrose synthesis since sucrose synthesis occurred in sh1 at the same rate as in wild type and sh2. Sucrose synthetase is greatly reduced in sh1; therefore, sucrose synthesis may occur through the action of some other enzyme or could be due to the residual 5% sucrose synthetase activity found in this sh1 allele.

The observations discussed above suggest that sucrose metabolism in sh1 is deficient in comparison to sucrose metabolism in wild type and sh2 and that sucrose synthetase plays a role in starch biosynthesis. These data are in agreement with the results obtained by Chourey and Nelson (1979) which suggest that sucrose synthetase is involved in sucrose breakdown. In addition to the results obtained from the [^{14}C] labelling experiments other data indicate the involvement of sucrose synthetase in starch biosynthesis. As shown earlier, the weight and starch content of wild type and sh2 kernels were greater when kernels were grown on sucrose whereas sh1 kernels were heavier and had greater

starch content when grown on reducing sugars. These results are consistent with the function of sucrose synthetase in the breakdown of sucrose. Superior growth of *sh1* seeds when grown on reducing sugars may be attributed to partially bypassing the block in sucrose metabolism caused by the lack of sucrose synthetase. By supplying reducing sugars to *sh1* kernels the block in sucrose metabolism may be bypassed allowing reducing sugars to be utilized for starch biosynthesis and resulting in kernels having greater starch content.

One intriguing observation, concerning the utilization of sugars, is that there is considerable synthesis of sucrose in kernels grown on reducing sugars. Why then are differences in seed growth observed due to carbon source? One might expect, *a priori*, that sucrose produced in wild type and *sh2* kernels grown on reducing sugars would be utilized in starch biosynthesis, by conversion of sucrose by sucrose synthetase, and thus negate any differences due to carbon source. Yet there exist differences in seed weight and starch content due to carbon source.

One explanation for the differences observed in wild type and *sh2* has to do with the rate at which sugar is transported into the endosperm tissue. In comparing the rate of sucrose and glucose transport using [^{14}C] labeled sugars, it was found that glucose was transported at a 20%

greater rate than sucrose in molar equivalents (Figures 37 & 38). But in hexose equivalents, sucrose supplies hexose as glucose at a 60% greater rate. Thus sucrose may be a superior carbon source due to the greater amount of carbon transported into the endosperm tissue.

Another explanation for the results found in wild type and sh2 kernels is the following. If the sucrose that is transported into the endosperm tissue is used in starch synthesis more efficiently than the sucrose that is synthesized internally, the differences can be explained. Delmar and Albersheim (1970) have suggested that sucrose synthetase is associated with the cell membrane in Phaseolus and that breakdown of sucrose by sucrose synthetase may occur as sucrose is transported into the cells. If a similar situation occurs in maize, then sucrose that is transported into the cells may be broken down by sucrose synthetase as it passes into the endosperm cells. Sucrose that is synthesized internally, as occurs in cells grown on reducing sugars, may be stored in vacuoles or other cell compartments and hydrolyzed to a greater extent by invertase than by sucrose synthetase.

SUMMARY

Kernels of wild type, sh1 (deficient in sucrose synthetase) and sh2 (deficient in ADPG pyrophosphorylase), placed in culture on sucrose at 5dpp, will develop to maturity and germinate. It was determined that development in vitro mimics in vivo development by all of the following criteria.

(1) The kernels have their expected phenotype at maturity and when germinated, vigor differences were observed that reflect differences seen in field grown seeds.

(2) The enzymes sucrose synthetase, ADPG pyrophosphorylase and starch synthetase were examined throughout development. These enzymes exhibit activity profiles similar to those found in field grown seeds. Sucrose synthetase was deficient in sh1 and ADPG pyrophosphorylase was deficient in sh2.

(3) Analysis of sugars revealed that there was a build up of sucrose in the mutants that is similar to the build up found in field grown maize.

(4) Relative differences were found in starch levels of kernels grown in vitro that are the same as kernels grown in vivo. Wild types seeds had the greatest starch while sh2 had the least.

After determination that in vitro kernel development reflects in vivo growth, kernels of all 3 genotypes were grown on sucrose, glucose or fructose. Analysis of the developing and mature seeds revealed that:

(1) Mature seeds of all genotypes exhibited their expected phenotype at maturity regardless of sugar source.

(2) Regardless of sugar source, sucrose, glucose and fructose were found in the endosperm tissue indicating that kernels are capable of synthesizing sucrose. Kernels grown on reducing sugars had greater amounts of reducing sugars than kernels grown on sucrose. Also, there was an increase in the concentration of sucrose, similar to field grown maize, in the mutants regardless of the sugar source.

(3) Wild type and sh2 seeds had a significantly greater weight and starch level when grown on sucrose than when grown on reducing sugars. In contrast to wild type and sh2, sh1 kernels had a greater weight and starch level

when grown on glucose. Futhermore, seed vigor was correlated with starch content and seed weight.

In an effort to determine the basis for the differences observed in the seeds grown on the different sugars, a series of pulse-chase experiments were conducted to examine the uptake and metabolism of [^{14}C] labeled sucrose and glucose, over a 5 hr. period, into kernels grown in vitro.

Evidence indicates that growth and metabolism differs in sh1 in comparison to wild type and sh2. These differences can be attributed to the lack of sucrose synthetase in sh1. Furthermore, evidence indicates that sucrose synthetase is involved in sucrose breakdown and not sucrose synthesis.

(1) It was found that 55% of the supplied sucrose was transported into the endosperm tissue without hydrolysis indicating that reducing sugars as well as sucrose are transported into the endosperm cells.

(2) The amount of [^{14}C] labeled sucrose taken up was similar in wild type and sh2 kernels. In contrast, uptake was less in sh1. This may be expected if sucrose breakdown is limiting and thus, less sucrose is transported into the endosperm cells.

(3) The conversion of sucrose was similar in wild type and sh2. Over the time period examined there was an

increase in the relative amount of labeled fructose found in relation to labeled glucose. There was more label in reducing sugars than in sucrose by the fifth hour. There was not a relative increase in fructose to glucose in sh1 and there was greater label in sucrose than in reducing sugars at the fifth hour. This indicates a deficiency in sucrose utilization in sh1, again suggesting the involvement of sucrose synthetase in sucrose breakdown. Also, the relative increase in fructose may be expected since fructose is one of the products of sucrose breakdown by sucrose synthetase.

(3) The uptake and conversion of labeled glucose was similar for the 3 genotypes. Sucrose was labeled at the same rate in the 3 genotypes indicating that no differences exist in glucose metabolism in the 3 genotypes.

All of these data suggest that the role of sucrose synthetase is in the breakdown, and not synthesis, of sucrose.

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BIOGRAPHICAL SKETCH

The author was born and reared in Evarts, Kentucky, a small town in the southeastern part of the state. As a child, he was very interested in science and established a lab at the age of 10. Little did he know that his childhood fascination with science would develop into his life's work. His interest in science increased, especially in biology, and he decided to major in biology as an undergraduate at The University of Alabama. He became fascinated with plant developmental biology and genetics and worked in the area of plant development for his master's degree at The University of Alabama. His Ph.D. work, at the University of Florida, was in the area of genetics and physiology of starch synthesis in maize. The author is presently a post-doctoral associate at Washington State University. He is married to C. L. Sheffield and claims to have no children. However, when pressed, he will admit to having a cat. He has never been to Paris.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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